Tu-Pos392

EFFECT OF MEMBRANE VOLTAGE ON THE PLASMA MEMBRANE H^+-ATPASE OF SACCHAROMYCES CEREVISIAE. Donna Seto-Young and David S. Perlin. Dept. of Biochemistry, The Public Health Research Institute, New York, N.Y, 10016.

The effects of membrane voltage on the plasma membrane H^+-ATPase from Saccharomyces cerevisiae were examined in a novel in vitro system that was capable of forming large interior positive membrane potentials. This membrane potential-generating machinery utilized the lipophilic electron carrier tetracyanoquinodimethane (TCNQ), located within the membrane bilayer, to mediate electron flow from vesicle-entrapped ascorbate to external ferricyanide. Membrane potentials formed in this system were followed by the potential-dependent probe oxonol V. A characteristic fluorescence quenching phase was observed which rapidly reached a steady-state ($T_{1/2}$ ~ 3 sec.) and which lasted for at least 90 s and then decayed with a $T_{1/2}$ ~ 180 sec. A maximum membrane potential of 250 mV was determined at the steady-state and was found to inhibit ATP hydrolysis by wild type H^+-ATPase reconstituted into liposomes from 34 to 46%. In contrast to wild type enzyme, membrane potential had little effect on a Ser_{368} —> Phe mutant enzyme (pma1-105) suggesting that it is defective in electrogenic proton translocation. Applied membrane voltage was also found to alter the sensitivity of wild type enzyme to vanadate at concentrations less than 50 μM. The data suggest a tight coupling between the charge-transfer and ATP hydrolysis domains and provide a new vehicle for studying the electrogenic properies of mutant enzymes.

Tu-Pos393

INHIBITION OF VANADATE-SENSITIVE H^+ATPASE ACTIVITIES FROM PLANT ROOTS BY FLUORESCAMINE AND ITS DERIVATIVES Shu-I Tu, Deidre Patterson, and David Brauer

Flourescamine (FL) labeling of KI washed corn root microsomes decreased the activities associated with the vanadate-sensitive H^+-ATPase of the plasma membrane. However, the ATP hydrolysis was much less sensitive than coupled proton pumping. When the microsomes were treated with 0.7 mM of FL, the proton pumping was essentially abolished but more than 50% of the ATP hydrolysis activity remained. While the effect was in proportion to the extent of total labeling, reaction with the non-phospholipid components occurred at lower FL concentrations. The use of pre-formed FL-primary amine derivatives also caused a differential inhibition to the coupled activities of the H^+-ATPase. The effectiveness of the derivatives to inhibit proton pumping increased with an increased solubility in the membranes. It has been shown that both the direct labeling and the partitioning of the derivatives resulted in the incorporation of an multiple H-bonding moiety in the membrane. Thus, the results suggest that the proton pumping may be indirectly coupled to the ATP hydrolysis and the coupling mechanism is sensitive to the presence of derivatized FL structure with multiple H-bonding capability in the plasma membrane.

Tu-Pos394

IDENTIFICATION OF A STIMULUS-ASSOCIATED PARIETAL CELL PROTEIN WHICH INTERACTS WITH H/K ATPASE. J. Cuppoletti, P. Huang and D. H. Malinowska. Dept. of Physiology & Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0576.

The gastric H/K ATPase, responsible for HCl secretion by the parietal cell, is potently inhibited by the natural polypeptide, melittin through direct interaction with the 95 kDa alpha subunit at a site within the cytoplasmic domain of the enzyme. To investigate the potential physiological significance of this polypeptide binding site on the H/K ATPase, we searched for an endogenous parietal cell protein with melittin-like determinants using an antimelittin antibody. An approximately 70 kDa protein (doublet) which shows immunoreactivity with antimelittin antibody on Western blots was found to be enriched in stimulated rabbit gastric vesicles and virtually absent from resting rabbit gastric vesicles, suggesting that the protein undergoes stimulus-dependent changes in association with the gastric H/K ATPase-containing membranes. The 70 kDa protein and melittin binding sites appear to overlap, based on the finding that melittin displaces the 70 kDa protein from stimulated vesicles. In studies using isolated purified rabbit gastric parietal cells, the 70 kDa protein exhibited time- and stimulus-dependent redistribution between the cytosol and a membrane fraction. Histamine-increased HCl accumulation (monitored with ^{14}C-aminopyrine uptake) correlated with movement of the 70 kDa protein from the cytosolic to the membrane fraction. With subsequent treatment with cimetidine (an H_2-blocker which inhibits histamine-stimulated HCl accumulation), the 70 kDa protein disappeared from the membranes and was found largely in the cytosolic fraction. Fractions from hog and rabbit gastric mucosae are being used as a source to isolate and purify the 70 kDa protein. Immunopurification using antibody coupled to Sepharose results in enrichment of the 70 kDa doublet. However, only the higher MW band of the 70 kDa doublet is enriched in stimulated vesicles and this band specifically binds to H/K ATPase-affinity supports. Thus, our hypotheses are that: (1) interaction of the endogenous 70 kDa protein (higher MW band of the doublet) occurs at a site which overlaps with the melittin binding site on the H/K ATPase, resulting in changes in its activity or location; and (2) this interaction plays an important physiological role in regulated HCl secretion by the gastric parietal cell. Supported by DK38808, Ohio AHA, Lilly Young Investigator Award & Cystic Fibrosis Foundation (JC); Ohio AHA (DHM).

Tu-Pos395

STUDIES ON FUNCTION AND STRUCTURE OF DISULFIDE BONDS IN GASTRIC H,K-ATPASE. Dar Chow, James Crothers, Jr., Kathy Browning, and John Forte. Dept. of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

The H,K-ATPase is the gastric proton pump that exchanges H^+ and K^+ across the apical membrane of the parietal cell. It is evolutionarily related to the Na,K-ATPase and has similar structure. The H,K-ATPase operates as a heterodimer consisting of a 114 kDa peptide, which has been called the catalytic α-subunit, and a heavily glycosylated β-subunit with a 34 kDa core peptide. The β-subunit has been cloned by several laboratories, including ours. The amino acid sequence suggests six cysteines are located in its extracellular domain. By analogy with the Na,K-ATPase, we tested the hypothesis that these cysteines form three disulfide bonds. Here we report effects of disulfide bond reduction on enzyme activity, which was monitored as K^+-stimulated ouabain-insensitive *p*-nitrophenylphosphatase (pNPPase) or ATPase. Incubation of H,K-ATPase-enriched gastric microsomes in 0.4M 2-mercaptoethanol (2ME) at 44°C inactivated the enzymatic activity, while 0.4M 2ME at 37°C or 0.4 M ethanol at 44°C had no effect on activity. Raising either temperature or 2ME concentration accelerated enzyme inactivation. At concentrations of 2ME lower than 0.23M, inactivation was not seen up to 51°C, above which the enzyme starts to be thermally inactivated. Interestingly, monovalent cations protected against inactivation by 2ME with the same relative effectiveness as their stimulation of the enzyme in absence of 2ME (i.e., Tl > K >> Na). Using the alkylating reagent, fluorescein-5-maleimide, specific for sulfhydryl groups, we detected an increase in free sulfhydryls in the β-subunit after 2ME treatment. These results demonstrate the contribution of disulfide bonds to the stability of the enzyme and are the first evidence that the β-subunit may be functionally linked to enzymatic activity. Studies are under way to characterize the disulfide bonds that are reduced. (Supported by DK38972 & DK10141)

Tu-Pos396

CALCIUM DISSOCIATION FROM PHOSPHORYLATED Ca^{2+}-ATPase TOWARDS THE SARCOPLASMIC RETICULUM LUMEN : A NONSEQUENTIAL MECHANISM FOR CALCIUM ION TRANSLOCATION.

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Using rapid filtration, we investigated the kinetics of dissociation towards the lumenal side of sarcoplasmic reticulum (SR) of the two Ca^{2+} ions transported by the Ca^{2+}-dependent ATPase of SR vesicles, previously made leaky with ionophore. First, we measured the rate at which ATPase approached steady-state after addition of ATP in the absence of potassium, at pH 6. Under these conditions, the observed kinetics did not reveal any very fast phase of $^{45}Ca^{2+}$ dissociation. Next, by isotopic dilution of calcium, we measured the kinetics of $^{45}Ca^{2+}$ dissociation under turnover conditions, in the presence of potassium at pH 6 or 7, and found that these kinetics were monophasic. Moreover, when they were measured in the presence of a high concentration of calcium designed to mimic a high concentration of calcium in the lumenal medium, they only departed slightly from monophasic behavior, both at pH 6 and pH 9. The absence of clearcut biphasic kinetics in the presence of a high concentration of calcium at alkaline pH, where the transport sites on the lumenal side are expected to be saturated, excluded the possibility that dissociation of the two transported Ca^{2+} ions towards the lumenal side of the vesicles was strictly sequential, as would be the case for calcium translocation through a narrow channel. The last method of dissociation rate measurement tested was designed to allow separate observation of the dissociation kinetics of each of the two Ca^{2+} ions bound to ATPase, after each had been labeled by partial isotopic exchange. This method was based on the fact that phosphorylation-induced Ca^{2+} occlusion was faster than Ca^{2+} dissociation from nonphosphorylated ATPase. We found that after ATP-induced phosphorylation, the two Ca^{2+} ions dissociated separately, with virtually identical rate constants. As a control, the partial isotopic exchange procedure allowed us to confirm that, in contrast, the two Ca^{2+} ions dissociated from nonphosphorylated ATPase towards the cytoplasmic medium at different rates. Our results suggest that after sequential binding to nonphosphorylated ATPase, these two ions are released to the lumenal side of the phosphorylated SR vesicles simultaneously.

Tu-Pos397

CHEMICAL MODIFICATION OF CYSTEINYL RESIDUES IN THE CA-ATPASE OF SARCOPLASMIC RETICULUM. A. Wawrzynow and J.H. Collins. Dept. Biol. Chem., School of Medicine, and Medical Biotechnology Center, Univ. of Maryland, Baltimore, MD 21201.

Thiol-specific structural probes were used for modification of Cys residues of Ca-ATPase of rabbit skeletal muscle sarcoplasmic reticulum. The probes used were the maleimide derivatives fluorescein-5-maleimide (FMal) and 4-dimethylaminophenylazophenyl-4'-maleimide (DABMal), and the acetamide derivative 4-dimethylaminophenylazophenyl-4'-iodoacetamide (DABIA). Sarcoplasmic reticulum membranes were reacted with a 1-, 10- or 100-fold molar excess of probe and extensively proteolyzed with trypsin. Soluble tryptic peptides were purified by reverse phase HPLC and subjected to sequence analysis in order to identify the labeled Cys residues. The extent and specifity of Cys labeling varied with the chemical properties and molar excess of the probe used. The relatively hydrophilic FMal mainly labeled several Cys residues in the "A1 fragment" (residues 199-505) of the Ca-ATPase, the more hydrophobic DABIA labeled the "B fragment" (residues 5061001), and DABMal labeled both parts of the enzyme. (Supported by NIH grant P01-HL-27867.)

Tu-Pos398

EFFECT OF cAMP, cGMP AND PROTEIN KINASE ACTIVATION ON RESTING CYTOPLASMIC AND DENSE TUBULAR CALCIUM LEVELS IN THE HUMAN PLATELET. J. Johansson, J. Tao, W.Jy and D.H. Haynes. Dept. Mol. Cell. Pharmacology, Univ. of Miami School of Medicine, Miami, FL 33101.

Studies from this laboratory have shown that the resting level of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) of the human blood platelet(measured by Quin-2) is the result of the balance between passive leakage and active extrusion via a Ca^{2+}-ATPase pump and a Na/Ca^{2+} exchanger located in the plasma membrane (PM). The pump, which makes the larger contribution, has a Km of 80 $\pm$ 10 nM and a Hill coefficient (n) of 1.7 $\pm$ 0.3. At resting $[Ca^{2+}]_{cyt}$ = 110 nM it exports Ca^{2+} at a rate equivalent to 63% of its Vm. The resting level of free Ca^{2+} in the dense tubule ($[Ca^{2+}]_{cyt}$ is determined by the balance of Ca^{2+} accumulation by a dense tubular (DT) Ca^{2+}-ATPase pump and passive leakage back to the cytoplasm. Chlorotetracycline (CTC) fluorescence is used to measure $[Ca^{2+}]_{dt}$. The presented data will show the following: (1) The Km, Vm and Hill coeffficients of the pumps and the rate constants for passive leakage can be determined independently, in situ, with fluorimetric indicators in intact platelets. (2) The Km of the DT pump is 180 $\pm$ 5 nM or approx. 2x that of the PM Ca^{2+}-ATPase. (3) cAMP increases the Vm of the PM pump, thereby decreasing $[Ca^{2+}]_{cyt}$; it increases Vm/Km of the DT pump, and on balance, increases $[Ca^{2+}]_{dt}$. (4) cGMP increases the Vm of the PM pump, does not alter the DT pump, decreases $[Ca^{2+}]_{cyt}$, thereby decreasing the $[Ca^{2+}]_{dt}$. (5) Phorbol ester has no effect on the PM pump or $[Ca^{2+}]_{cyt}$, but increases Vm/Km of the DT pump, thereby increasing $[Ca^{2+}]_{dt}$. (6) Both algebraic and computer modelling shows that the observed changes in resting $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{dt}$ are predicted by the above changes in Vm and Km. Observation (4) explains the anti-aggregatory and anti-thrombotic efforts of sodium nitroprusside on platelets. Observation (3) offers an explanation as to why antiplatelet drugs such as dipyridamole give variable results. Support: NIH HL 38228 and HL 07188.

Tu-Pos399

The Mg^{2+}-ATPase of T-tubules from skeletal muscle forms an alkaline-stable phosphoenzyme intermediate (E~P). Norbert W. Seidler and Anthony Martonosi (Intro. by Troy J. Beeler), Dept. of Biochemistry, SUNY Health Science Center, Syracuse, New York 13210.

The Mg^{2+}-ATPase of transverse tubule membranes from rabbit skeletal muscle contributes to the "basal" Ca^{2+}-independent ATPase activity of sarcoplasmic reticulum preparations. The Mg^{2+}-ATPase has low affinity for ATP and requires millimolar concentrations of Mg^{2+}, but no Ca^{2+} for activity. The rate of ATP hydrolysis in the presence of 2.0-5.0 mM ATP, 5.0 mM Mg^{2+}, 2.0 mM EGTA at pH 6.8 and 24°C was 0.18 μmoles $mg^{-1}\cdot min^{-1}$ in sarcoplasmic reticulum vesicles and 0.84 μmol $mg^{-1}\cdot min^{-1}$ in T-tubule preparations isolated according to Rosemblatt and Scales (Mol. Cell. Biochem. 87, 57-69, 1989). The hydrolysis of ATP was accompanied by the formation of an acid-labile, alkali-stable phosphoprotein intermediate (E~P) from [^{32}-P]ATP. Since the E~P of Mg^{2+}-ATPase rapidly decomposed under the acidic conditions used for the assay of the phosphoenzyme intermediate of Ca^{2+}-ATPase, the Penefsky column-centrifugation procedure (Meth. Enzymol. 56, 527-530, 1979) was used at alkaline pH (pH ≈13) for the separation of the [^{32}-P]-labeled proteins. The steady state concentration of the alkaline-stable E~P increased from 1.8 to 6.5 nmoles/mg protein as the ATP concentration was raised from 1 to 5 mM in the presence of 5 mM $MgCl_2$ and 0.5 mM EGTA. After polyacrylamide gel electrophoresis at alkaline pH and transfer of proteins to Immobilon membranes, several [^{32}-P]-labeled bands were seen on the autoradiograms, with a protein of approximately 30 kDa showing the highest radioactivity.

This study suggests that a membrane protein from T-tubules associated with Mg^{2+}-ATPase activity forms an alkaline-stable phosphoenzyme intermediate during catalysis. The pH dependence of E~P hydrolysis is consistent with E~P being phosphohistidine. (Supported by a fellowship from the NIAMS and by research grants to Dr. A. Martonosi from the NIH, NSF and MDA)

Tu-Pos400

CALCIUM ACTIVATED ATPASE OF *SCHISTOSOMA MANSONI* TEGUMENT.

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A calcium dependent ATPase activity is characterized in the tegumental fraction of *Schistosoma mansoni*. The activity is measured in the presence of azide and ouabain at pH 7 and typical maximal values of 150 nmol $Pi.mg^{-1}.min^{-1}$ at 37° are obtained. Maximal activity is obtained with 1 μM Ca^{2+} in the presence of 5 μM free Mg^{2+}. High millimolar Mg^{2+} totally inhibits the calcium-activated enzyme. In the absence of Mg maximal calcium-dependent activity is obtained only at 100 μM Ca^{2+}. In the absence of calcium, the Mg^{2+} activated ATPase reaches maximal values of 40 nmol $Pi.mg^{-1}.min^{-1}$ at 5 μM Mg^{2+} and remains constant up to millimolar Mg^{2+}. The ion dependence profile of this enzyme is similar to that of plasma-membrane calcium-ATPase, recently described in the literature.

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Tu-Pos401

KINETIC EVIDENCE FOR TWO NUCLEOTIDE SITES ON THE CALCIUM PUMP OF SARCOPLASMIC RETICULUM

Richard J. Coll and Alexander J. Murphy, Department of Biochemistry, University of the Pacific, San Francisco, CA 94115

The calcium ATPase of sarcoplasmic reticulum exhibits complex kinetics when the velocity is measured as a function of ATP concentration. This behavior is also seen in other ion transport ATPases. The calcium pump shows a high affinity Km in which virtually all the enzyme (4-5 nmol/mg) is in the form of a steady-state phosphorylated intermediate (EP), and further increases of substrate concentration (mM) cause a 3-4 fold increase in rate. The way in which this second ATP binds to the EP is the basis for the present work, i.e., does mM ATP bind to the phosphorylated active site or to a separate allosteric site? We approached this by studying how mM ATP affects the behavior of the only substance unequivocally known to bind to the phosphorylated active site, the product ADP. The CaATPase was reacted with [γ-^{32}P]ATP to form the covalent EP intermediate. Noncompetitive inhibition by reactive red-120 and chelation of calcium allowed us to monitor single turnover kinetics of EP reacting with water or added ADP at 0°C. When ADP was added and the amount of product, [γ-^{32}P]ATP, formed was measured we found that added cold ATP did not interfere with EP reacting with ADP. We conclude that ATP cannot bind where ADP binds, the phosphorylated active site. This implies that when ATP at high concentrations causes an acceleration of EP hydrolysis, it must do so by binding to an allosteric site. Considering the monoexponential nature of product formation we observed, simple one-nucleotide site models cannot account for the above result. (Supported by NIH GM31083)

Tu-Pos402

SARCOPLASMIC RETICULUM CALCIUM ATPase: REACTION of A CARBODIIMIDE ADDUCT OF ATP AT ITS ACTIVE SITE.

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ATP-EDC, an adduct of a carbodiimide and ATP, was synthesized. Despite its limited stability, it was found to react with and inactivate the calcium ATPase of sarcoplasmic reticulum in its vesicular, nonionic detergent-solubilized and purified forms. The ADP form of the adduct was ineffective. Saturation kinetics, with an ATP-EDC concentration dependence midpoint in the 10 uM range, was observed, suggesting an active site affinity which is similar to that of ATP. The reaction was specific in that inactivation required reaction of about one adduct per ATPase. The modified enzyme could no longer be phosphorylated by ATP or Pi or hydrolyze p-nitrophenylphosphate, but retained the ability to undergo the high affinity calcim-dependent fluorescence change. It also bound trinitrophenyl-ADP and other nucleotides at least tenfold more weakly than the unmodified ATPase. The inactivation reaction required the presence of Mg^{2+} and Ca^{2+}, and was prevented by nucleotides such as ATP and ADP. For magnesium the inactivation-enabling effect occurred with a midpoint of 3mM. In the case of calcium the transition resembled high affinity binding in that it occurred cooperatively with a midpoint in the micromolar range. PAGE demonstrated that the reaction converted the ATPase (M_r=1.1x10^5) to a species with an apparent M_r=1.7-1.8x10^5. Since nonionic detergent-solubilized ATPase and purified ATPase gave similar results, intramolecular cross-linking is implicated. (Supported by NIH GM31083)

Tu-Pos403

Effect of solutes on the stacking of crystalline sheets of the Ca^{2+}-ATPase.

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The Ca^{2+}-ATPase crystals formed in detergent solubilized sarcoplasmic reticulum (SR) at 2°C in a crystallization medium (CM) of 0.1 M KCl, 10 mM K-MOPS, pH 6.0, 3 mM $MgCl_2$, 3 mM NaN_3, 5 mM DTT, 25 IU/ml Trasylol, 2 μg/ml 1,6-di-tert-butyl-p-cresol, 20% glycerol and 20 mM $CaCl_2$ (J. Biol. Chem. 263, 5277, 5287, 1988) contain highly ordered sheets of ATPase molecules, that associate into large multilamellar stacks (>100 layers) with defective packing in the third dimension. When the crystallization is performed in the same medium but in the presence of 40% glycerol at subzero temperature the stacking is dramatically reduced (≈4-5 layers) and the average diameter of the crystalline sheets is increased from 1.67 μm to 2.89 μm, providing improved conditions for electron microscope reconstruction of the Ca^{2+}-ATPase structure. Glycerol and low temperature presumably reduce stacking by interfering with the interactions between the hydrophilic headgroups of Ca^{2+}-ATPase molecules in adjacent lamellae, while not affecting or promoting the ordering of ATPase molecules within the individual sheets. Addition of Na-propionate or NH_4-acetate (0.4-0.8 M) to the crystallization medium or an increase in the KCl concentration to 0.4-0.8 M severely impaired crystallization. Replacement of 40% glycerol with 10-50% glucose or supplementation of CM with polyethyleneglycol (PG 3000 or 8000; 1, 2, 5 and 8%) had no beneficial effect on the order of crystalline arrays compared with media containing 40% glycerol. (Supported by research grants from the NIH, NSF and MDA)

Tu-Pos404

Covalent photolabeling of the cytoplasmic or luminal domains of Ca^{2+}-ATPase with fluorescent azido-dyes. Elek Molnar and Anthony Martonosi (Intro. by Thomas J. Csermely), Dept. of Biochemistry, SUNY Health Science Center, Syracuse, New York 13210.

Sarcoplasmic reticulum (SR) vesicles were incubated with azido derivatives of Cascade Blue (ACB), Lucifer yellow (ALY), 2,7 naphthalene disulfonic acid (ANDS), and fluorescein (AF) for 0.1-24 hours at 2°C. All four dyes gave intense reaction with the cytoplasmic domain of the Ca^{2+}-ATPase on photoactivation after brief incubation. The penetration of the dyes into the luminal space of the SR was determined after centrifugation through Sephadex microcolumns to remove the external dye, followed by photolabeling and gel electrophoresis of the photolabeled proteins. The reaction of ACB and ANDS with the Ca^{2+}-ATPase and with calsequestrin increased progressively during incubation up to 24 hours indicating their slow accumulation in the luminal space, while ALY and AF did not show significant penetration into the vesicles.

The distribution of the covalently attached ACB in the Ca^{2+}-ATPase was tested by tryptic proteolysis after labeling exclusively from the outside (OS), from the inside (IS) or from both sides (BS). In all cases intense ACB fluorescence was seen in the A fragment with inhibition of ATPase activity. In the OS preparations the A_1, while in IS the A_2 fragment was more intensely labeled. There was no significant incorporation of ACB into the region of B fragment identified by FITC fluorescence. The crystallization of the Ca^{2+}-ATPase by EGTA + decavanadate was inhibited in the OS but not affected in the IS preparations.

The total amount of ACB photoincorporated into SR proteins after incubation for 24 hours was 1.75 nmoles/mg protein; 2/3 of this labeling occurred from the outside and 1/3 from the inside. Similar level of labeling was obtained in media that stabilize the E_1 or the E_2 conformation of the Ca^{2+}-ATPase. (Supported by research grants from the NIH, NSF and MDA)

Tu-Pos405

Orientations of Ca^{2+}-ATPase, phospholipids and trehalose in multilayers of sarcoplasmic reticulum probed by polarized infrared ATR spectroscopy (P-IR-ATR). Rene Buchet, Dept. Biochemistry, SUNY Health Science Center, Syracuse, New York 13210.

Dichroic ratios were obtained from the P-IR-ATR spectra of thin dry multilayers of sarcoplasmic reticulum containing ~7.5 μg protein/cm^2, deposited either on the Ge or on the ZnSe ATR plates. The dichroic ratio of the CH_2 vibrations (2923 cm^{-1}) of extracted sarcoplasmic reticulum phospholipids on Ge plate is 1.56, compared with a dichroic ratio of 1.68 obtained on dry films of whole sarcoplasmic reticulum; the corresponding mean angles between the phospholipid hydrocarbon chain axis and the normal of the plane are 43° in extracted phospholipids and 46° in the whole sarcoplasmic reticulum, respectively. A random orientation would give a mean angle of 54.7°. The greater disorder in the hydrocarbon chain region of the whole sarcoplasmic reticulum may be related to protein-lipid interactions. The dichroic ratios of the Amide I band (1650 cm^{-1}) of the Ca^{2+}-ATPase in the Ca_2-E_1 state and in the EGTA and vanadate stabilized V-E_2 state were nearly identical (1.60 vs. 1.62); this implies that the changes related to transition between the Ca_2-E_1 state and the V-E_2 state can not be detected by P-IR-ATR. The dichroic ratio of the C-C (1033 cm^{-1}) or C-O stretching band (1046 cm^{-1}) of trehalose incorporated into SR films was 1.2 on Ge plate; this corresponds to a mean angle of ~70° between the plane of the trehalose ring and the normal of the film plane, suggesting that the trehalose molecules are surprisingly well oriented in the polar headgroup region of the phospholipids. The orientation of the trehalose was not affected by the presence of Ca^{2+}-ATPase. (Supported by a fellowship from the American Heart Association NY State Affiliate and by grants to A. Martonosi from the NIH, NSF and MDA)

Tu-Pos406

THE REACTION CYCLE OF THE SARCOPLASMIC RETICULUM Ca^{2+}-ATPase WITH Sr^{2+} BOUND TO THE TRANSPORT SITES. Taro Fujimori and William P. Jencks. Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254

The binding of Ca^{2+} or Sr^{2+} is known to cause a conformational change in the SR Ca^{2+}-ATPase that allows phosphoenzyme formation from ATP. The ionic radius of Sr^{2+}, 1.3 Å, is larger than that of Ca^{2+}, 1.1 Å. The $K_{1/2}$ of ~100 μM for the binding of Sr^{2+} to the transport site is ~50-fold larger than the $K_{1/2}$ of 2 μM for Ca^{2+} (pH 7.0, 25°C, 5 mM Mg^{2+}, 0.1 M KCl). However, the observed rate constant of 220 ± 25 s^{-1} for phosphoenzyme formation is the same with Ca^{2+} or Sr^{2+} bound to the transport sites (0.4 mM ATP). These data indicate that the conformational change is a result of the bound ions acting as a switch and not a result of the bound ions actively pushing on the enzyme because the observed rate constant for phosphorylation is not sensitive to the size of the ion. The dissociation of both Sr^{2+} and Ca^{2+} from the lumenal transport site, measured by the rate of phosphoenzyme hydrolysis, occurs with the same rate constant of 19 ± 4 s^{-1}. The rate constant of 110 ± 40 s^{-1} for dissociation of Sr^{2+} from $^{c}E{\cdot}ATP{\cdot}Sr_2$ is comparable to the rate constant of ~80 s^{-1} for dissociation of Ca^{2+} from $^{c}E{\cdot}ATP{\cdot}Ca_2$. The rate constants for the dissociation of Sr^{2+} and Ca^{2+} are similar from $E{\cdot}P{\cdot}Me_2$ and also from $^{c}E{\cdot}ATP{\cdot}Me_2$, despite large differences in binding affinity and ionic radii of the two ions, suggesting that these off rates are controlled by a process that is intrinsic to the protein.

Two mixed species of enzyme with both Sr^{2+} and Ca^{2+} bound to the transport sites were formed. The dissociation of Ca^{2+} from the cytoplasmic transport site is known to be sequential: the "outer" Ca^{2+} dissociates rapidly, with a rate constant of 60 s^{-1}, while dissociation of the "inner" Ca^{2+} is inhibited by external Ca^{2+}. $^{c}E{\cdot}Sr{\cdot}Ca$, which represents enzyme with Sr^{2+} bound to the "inner" site and Ca^{2+} bound to the "outer" site, was formed by incubating the enzyme with 730 μM Sr^{2+} and adding 190 μM $^{45}CaCl_2$ (10 mM $MgCl_2$, 0.1 M KCl). The binding of $^{45}Ca^{2+}$ to the transport sites was assayed by measuring the internalization of ^{45}Ca after the addition of ATP and EGTA. The binding of ^{45}Ca to $^{c}E{\cdot}Sr{\cdot}Sr$ is biphasic: the "outer" Sr^{2+} is replaced by ^{45}Ca rapidly, with a rate constant of 120 ± 40 s^{-1}, while the "inner" Sr^{2+} is replaced by ^{45}Ca slowly, with a rate constant of ~0.7 s^{-1}. $^{c}E{\cdot}Ca{\cdot}Sr$, which represents enzyme with Ca^{2+} bound to the "inner" site and Sr^{2+} bound to the "outer" site, was formed by incubating the enzyme in 30 μM $^{45}CaCl_2$ and adding 300 μM $SrCl_2$. The dissociation of ^{45}Ca from $^{c}E{\cdot}^{45}Ca{\cdot}^{45}Ca$ in the presence of 300 μM $SrCl_2$ is biphasic: the "outer" ^{45}Ca dissociates with a rate constant of >30 s^{-1} while the "inner" ^{45}Ca dissociates with a rate constant of 4 ± 1 s^{-1}. The addition of ATP and EGTA to $^{c}E{\cdot}Sr{\cdot}^{45}Ca$ and $^{c}E{\cdot}^{45}Ca{\cdot}Sr$ results in the internalization of one ^{45}Ca/EP and, presumably, one Sr/EP.

Tu-Pos407

The effects of pressure on the conformation and activity of sarcoplasmic reticulum Ca^{2+}-ATPase. Istvan Jona and Anthony Martonosi (Intro. by W. D. Stein), Dept. of Biochemistry, SUNY Health Science Center, Syracuse, New York 13210.

High pressure (150 MPa) increases the intensity and polarization of fluorescence of FITC labeled Ca^{2+}-ATPase in a medium containing 0.1 mM Ca^{2+}, suggesting a reversible pressure-induced transition from the E_1 into an E_2-like state with dissociation of ATPase oligomers. Under similar conditions but using native SR vesicles, high pressure caused the reversible release of Ca^{2+} from the high affinity Ca^{2+} sites of Ca^{2+}-ATPase, as indicated by changes in the fluorescence of the Ca^{2+} indicator, Fluo-3; this was accompanied by reversible inhibition of the Ca^{2+}-stimulated ATPase activity measured in a coupled enzyme system of pyruvate kinase and lactate dehydrogenase, and by redistribution of PRODAN in the lipid phase of the membrane, as shown by marked changes in its emission characteristics.

In a Ca^{2+}-free medium the fluorescence intensity of FITC-ATPase was not affected or only slightly reduced by high pressure. The enhancement of TNP-AMP fluorescence by 100 mM inorganic phosphate in the presence of EGTA and 20% dimethylsulfoxide was essentially unaffected by 150 MPa pressure at pH 6.0 and was only slightly reduced at pH 8.0. The enhancement of TNP-AMP fluorescence by Pi is associated with the Mg^{2+}-dependent phosphorylation of the Ca^{2+}-ATPase by Pi with the formation of $Mg{\cdot}E_2$-P intermediate, a reaction associated with the E_2 state.

These observations suggest that high pressure stabilizes the enzyme in an E_2-like state characterized by low reactivity with ATP and high reactivity with Pi. The transition from the E_1 to the E_2-like state involves a decrease in the effective volume of Ca^{2+}-ATPase. (Supported by research grants from the NIH, NSF and MDA)

Tu-Pos408

INFRARED SPECTROSCOPIC DETECTION OF CONFORMATIONAL CHANGES IN THE CATALYTIC CYCLE OF SARCOPLASMIC RETICULUM ATPASE

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Fourier transform infrared spectroscopy was used to investigate conformational changes in the Ca^{2+}–ATPase of sarcoplasmic reticulum during the catalytic cycle. The ATPase reaction was started in the infrared cuvette by release of ATP from the inactive, photolabile ATP–derivative caged ATP. Absorption spectroscopy in the visible spectral region using the Ca^{2+}–sensitve dye Antipyrylazo III ensured that the infrared samples were able to transport Ca^{2+} in spite of their low water content, which is required for mid infrared measurements (1800–950 cm^{-1}). Small, but characteristic and higly reproducible infrared absorbance changes were observed upon ATP release. These infrared absorbance changes exhibit different kinetic properties. Comparison to model compound infrared spectra indicates that they are related to photolysis of caged ATP (1540–950 cm^{-1}), hydrolysis of ATP (1300–1000 cm^{-1}) in consequence of ATPase activity and to conformational changes in the active ATPase (1800–1500 cm^{-1}). The absorbance changes due to conformational alterations in the ATPase were observed in the region of Amide I and Amide II protein absorbance and probably reflect the molecular processes upon phosphoenzyme formation. Since the absorbance changes were small compared to the overall ATPase absorbance, no major rearrangement of ATPase conformation as the result of catalysis could be detected.

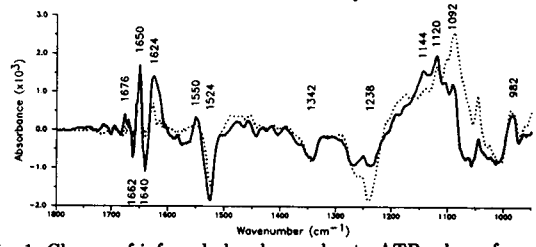

Fig. 1. Change of infrared absorbance due to ATP release from caged ATP in an ATPase sample at 0° C. Spectra recorded 4 s (full line) or 2 min (dotted line) after ATP–release.

Tu-Pos409

INTERACTION OF THE VOLTAGE-SENSITIVE DYE RH-421 WITH (Na,K)-ATPase. Blanche Schwappach, Walter Gassman, and P. A. George Fortes. Department of Biology, University of California San Diego, La Jolla, CA 92093-0116.

The fluorescence of styryl dyes (e.g. RH-421) is sensitive to conformational changes in (Na,K)-ATPase (Klodos & Forbush, 1988 J. Gen. Physiol. 92:46a, Sturmer, et al., 1990 ibid. 96:75a). In order to characterize the site(s) of interaction of the dye with the enzyme, we measured spectra and lifetimes of RH-421 bound to purified canine renal (Na,K)-ATPase and in model systems, and determined resonance energy transfer between the dye and fluorescent probes on the enzyme. RH-421 was an efficient acceptor for resonance energy transfer from anthroylouabain bound to the ouabain site and from 5-iodoacetamidofluorescein-labelled cysteine. Lifetime measurements showed at least two populations: 2.2 ns (63%) and 0.7 ns (37%) for RH-421 bound to (Na,K)-ATPase. These populations had different spectra. RH-421 bound to Bovine Serum Albumin had similar spectra and identical lifetimes as in (Na,K)-ATPase. By contrast, RH-421 had homogeneous spectra and a single lifetime in solvents (1 ns in ethanol, 1.78 ns in $CHCl_3$) and in anionic or cationic detergent micelles (0.77 ns); RH-421 had lifetimes ranging from 0.2 to 1 ns in sonicated dioleylphosphatydylcholine liposomes. The results suggest that RH-421 interacts with the (Na,K)-ATPase protein as well as with the lipid bilayer.

(Supported by ONR-N00014-88-K-0324)

Tu-Pos410

Digestion of Renal Na/K-ATPase by Non-selective Proteases with Preservation of Rb Occlusion. J.Capasso*, S.Hoving, and S.J.D.Karlish, Department of Biochemistry, Weizmann Institute of Science, Rehovot *(on leave from CONICET, Argentina.

We have recently reported that extensive tryptic digestion of Na/K-ATPase in the presence of Rb and absence of Ca ions, removes about half the protein, and produces membranes containing a stable 19Kd C-terminal and smaller fragments of the alpha chain, a largely intact beta chain, but Rb and Na occlusion are preserved(Karlish etal PNAS 87, 4566 (1990).

We have now extended these observations and show that extensive digestion with totally non-selective fungal proteases (pronase and proteinase-K) can remove up to 70 % of membrane protein without destroying Rb occlusion. A fragment of 19Kd appears (that from pronase having as N-terminal, Asn 830, as found with trypsin), but the beta chain is destroyed. At intermediate stages 21Kd (with pronase, having as N-terminal, lys 31) or 28 Kd (with proteinase-K) fragments are observed. These can be digested further to leave membranes with the 19Kd fragment as the largest polypeptide, but still intact Rb occlusion. For pronase, the specific digestion pattern and preservation of occlusion depends on the absence of divalent metal ions (Mg or Ca) and presence of Rb or Na ions during digestion.

The experiments suggest strongly that Rb occlusion sites are located within trans-membrane segments, including those of the crucial 19Kd fragment. The bulk of protein mass of the beta chain is not involved. The extraordinary resistance of the 19Kd fragment to further digestion, in the specific conditions, shows that it is compact (probably within the membrane) and innaccessible to essentially any protease. Thus non-selective proteases are promising tools which may permit identification and isolation of the minimal protein structures responsible for occlusion of cations.

Tu-Pos411

DIFFERENTIAL SENSITIVITY TO CALPAIN DIGESTION OF THREE ISOFORMS OF THE Na,K-ATPase. T. Cova and K.J. Sweadner. (Intro. by J.W. Peterson) Massachusetts General Hospital, Boston, MA 02114 and Dept. of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA.

There are three isoforms of the catalytic subunit of the Na,K-ATPase (α1, α2, and α3), M_r about 100,000. Previous work showed that the isoforms had markedly different sensitivities to digestion by trypsin, despite 85% sequence identity (Urayama and Sweadner 1988, BBRC 156: 796). In the present study, we investigated whether there is a differential sensitivity to digestion by calpain that might play a role in the regulation of protein turnover in vivo. Digestion of partially-purified Na,K-ATPase from rat brain and brainstem axolemma was carried out in vitro. The Na,K-ATPase subunits and their proteolytic fragments were detected by isoform-specific monoclonal antibodies on Western blots. In vitro conditions of calpain activation permitted a brief burst of activity, followed by auto-inactivation of the protease, as measured with casein as substrate. Under the same conditions, the α1 isoform of the Na,K-ATPase was not detectably digested. A small proportion of the α2 isoform was digested, and fragments of 40-60K, labeled by antibody, were seen. A larger proportion of the α3 isoform was digested, but no fragments stained by either of two antibodies were detected. This pattern of sensitivity differs from that seen with trypsin or chymotrypsin, in which case the α3 isoform is least sensitive and the α2 isoform is most sensitive. Other investigators have shown that the conformation of the Na,K-ATPase significantly affects whether or not tryptic cleavage occurs at certain sites, presumably due to substantial movements of the sites relative to the surface of the protein. With calpain, we found that in K^+ (the E2 conformation), α2 was more sensitive to digestion than in Na+ (E1). Supported by HL 36721.

Tu-Pos412

STRUCTURE OF THE Na,K-ATPASE STUDIED BY EPITOPE MAPPING. E.A. Aristarkhova, T. Cova, and K.J. Sweadner. Neurosurgical Research, Massachusetts General Hospital, Boston, MA 02114 and Dept. of Cellular and Molecular Physiology, Harvard Medical School.

The transmembrane folding of the catalytic subunit of the Na,K-ATPase has been predicted from analysis of the cDNA sequence, but more structural information is needed to test the model. Sensitivity to proteolysis has been used here as a tool to map the location of antibody binding sites. Controlled tryptic cleavage in Na^+ or K^+; chymotryptic cleavage in Na^+; and N-chlorosuccinimide cleavage at tryptophan have been used to generate defined fragments for mapping an antibody which recognizes denatured enzyme (McBX3). The antibody is selective for α3 but crossreacts detectably with α1 and the H,K-ATPase. The epitope mapped somewhere to the C-terminal side of the α1 chymotryptic site at aa 273, of the tryptophan at 418, and of the tryptic site at 435. More extensive tryptic cleavage in the presence of Rb^+ and EDTA, which removes most of the intracellular hydrophilic segments but leaves the transmembrane segments and a 19 kD C-terminal fragment (Karlish et al., 1990 PNAS 87: 4566), was used to further refine the location. McBX3 binding was removed, indicating that the site lies within the digested hydrophilic regions. This places the epitope most likely within the largest cytoplasmic domain. Other monoclonal antibodies VG_4, VG_2, and IIC_9 bound to the cleaved ("19 kD" shaved) preparation, consistent with the location of epitopes determined previously by sequence analysis of purified fragments. Cleavage of enzyme with V8 protease destroyed the binding site for VG_4, an antibody previously shown to bind to a tryptic fragment encompassing transmembrane segments H3 and H4. Since VG_4 binds to intact cells from the extracellular surface, the H3-H4 junction containing two glutamate residues seems the most likely epitope location. Supported by HL 36271.

Tu-Pos413

TRANSIENT AND STATIONARY Na/K PUMP CURRENTS IN GUINEA PIG VENTRICULAR MYOCYTES AT EXTERNAL SODIUM CONCENTRATIONS UP TO 250 mM.

Georg A. Nagel, Munthe Suenson, Masakazu Nakao, and David C. Gadsby (Introduced by Erich Windhager). *Laboratory of Cardiac Physiology, The Rockefeller University, New York, NY 10021.*

In squid giant axons, *Xenopus* oocytes, and guinea pig ventricular myocytes, stationary Na/K pump current is relatively constant at positive potentials but declines steeply at negative potentials. The steepness of that pump current-voltage (I-V) relationship at negative potentials is strongly influenced by the external Na concentration (Na_o) and, in guinea pig myocytes, it is reduced when Na_o is decreased from its physiological level of 145 mM. In the absence of external K, the Na/K transport cycle is interrupted and stationary pump currents are abolished: but, under those conditions, voltage pulses elicit transient pump currents which arise instantaneously and decay with voltage-dependent single-exponential time courses. We have determined both stationary and transient strophanthidin-sensitive (0.5 - 2 mM) currents, at Na_o up to 250 mM, in myocytes internally dialyzed and voltage-clamped using wide tipped (~5 μm), low resistance (~1 MΩ) pipettes and an intra-pipette perfusion device. The holding potential was set at 0 mV and the compositions of internal (pipette) and external solutions chosen to minimize ion channel and Na/Ca exchange currents. Currents were recorded, at ~36 °C, using 100 ms voltage pulses to potentials between -100 and +100 mV. When Na_o was raised from 150 mM to 250 mM, the slope of the pump I-V relationship steepened, i.e. the voltage dependence of the stationary pump current at negative potentials was increased. The rate constants of exponential decay of the transient pump currents in the negative voltage range were elevated by raising Na_o above 150 mM, and were reduced by lowering Na_o towards zero. The rate constants at positive potentials were relatively unaffected, implying a shift of the rate constant-voltage curve to more positive potentials with increasing Na_o. Correspondingly, the mid-point of the curve relating the quantity of moved charge to membrane potential was shifted to more positive potentials as Na_o was increased. Addition of 100 mM N-methylglucamine-Cl to the external solution containing 150 mM NaCl did not affect stationary or transient pump currents.

(Supported by NIH HL-36783 and the Irma T. Hirschl Trust.)

Tu-Pos414

DELIVERY OF ION MOTIVE ATPASES FROM NATIVE MICROSOMAL-RICH SOURCES INTO THE MAMMALIAN RED BLOOD CELL MEMBRANE. Scott Munzer, John Silvius and Rhoda Blostein. Departments of Medicine and Biochemistry, McGill University, Montreal, Quebec Canada.

It is now clear that several ion motive ATPases exist in multiple molecular forms. Thus, three tissue-specific isoforms of the catalytic alpha subunit of the Na,K-ATPase have been identified. Although the functional significance of the multiple isoforms remains to be determined, it is evident that the behavior of the same isozyme may vary from tissue to tissue. For example, there are differences in kinetic behavior of the kidney and red cell Na,K-ATPases, both of which are known to have the same catalytic isoform (α1). A plausible explanation is that the enzyme may be regulated by components of the membrane environment, i.e. specific lipids or proteins. To address this question, we have used polyethylene glycol (PEG)-mediated fusion to deliver ion pumps from one type of membrane (microsomes) into another (mammalian red cells). Experiments involving delivery of the sarcoplasmic reticulum (SR) Ca-ATPase into human erythrocytes were first carried out to assess the feasibility of the fusion protocol. Whereas there was little detectable Ca^{2+} uptake into control cells in either the absence or presence of extracellular ATP, a marked uptake of Ca^{2+} was observed in cells fused with SR Ca-ATPase. The higher level of Ca^{2+} uptake into SR microsome-fused red cells and the very long half-times observed for this process are consistent with transfer of functional Ca-ATPase into red cell membranes rather than mere adsorption of SR microsomes on the cell surface. We have also fused Na,K-ATPase from native dog kidney microsomes with human, sheep and dog red cells, the latter having the advantage of very low endogenous Na,K-ATPase activity. True delivery of pumps into the red cell membrane has been demonstrated by ouabain-sensitive Rb^+ uptake into cells energized by intracellular ATP as well as by ATP-stimulated Na^+ influx into and Rb^+ efflux from inside-out membrane vesicles prepared from the fusion-treated cells. (Supported by the Medical Research Council of Canada).

Tu-Pos415

RECRUITMENT OF AN INTERNAL POOL OF MATERNAL SODIUM PUMPS DURING EARLY DEVELOPMENT OF *XENOPUS LAEVIS*.

Pia Kuhl, Kathi Geering*, and Günther Schmalzing. Max-Planck-Institut für Biophysik, Frankfurt/M., FRG; *Institut de Pharmacologie de l'Université de Lausanne, Switzerland. (Intro. by David M. Mueller)

Xenopus eggs ready for fertilization have a large pool of intracellular sodium pumps, whereas the cell surface is devoid of sodium pumps (Schmalzing et al., Am. J. Physiol. 258, C179-C184, 1990). To examine the fate of the internal pool of maternal sodium pumps after fertilization, ouabain binding was determined in intact and detergent-permeabilized *Xenopus* embryos. Starting at the one-cell stage with virtually zero, the number of sodium pumps in plasma membranes turned out to rise with each cell cycle in expense of the number of internal sodium pumps. The total number of sodium pumps did not change from the unfertilized egg up to the 64-cell stage, suggesting that endowment of interblastomeric plasma membranes with sodium pumps depends completely on the recruitment of the maternally-derived pool of sodium pumps. At the 64-cell stage, about half of the internal sodium pumps were inserted into plasma membranes. Since ouabain binding sites could not be detected on the outer surface of intact embryos, sodium pumps seem to be solely inserted into interblastomeric plasma membranes. Selective appearance of sodium pumps in newly formed plasma membranes was confirmed by indirect immunofluorescence using an antibody against a fusion protein which contained 98 amino acids of the α1 subunits of the sodium pump of *Xenopus laevis* kidney cells (Ackermann and Geering, FEBS Lett. 269, 105-108, 1990). In sections of paraffin-embedded embryos all interblastomeric plasma membranes showed intense immunofluorescence, while the outer surface of embryos remained unlabeled. An increase in the total number of sodium pumps was observed after the morula stage and could be partially inhibited with cycloheximide in concentrations of 0.1-1 μg/ml thought not to affect cell division. In addition, cycloheximide reduced the translocation of internal sodium pumps to plasma membranes. Inhibition of formation of new membranes by blocking cytokinesis with cytochalasin B abolished the rise in the total number of sodium pumps. These findings suggest that the net increase in the number of sodium pumps by protein synthesis and the insertion of internal sodium pumps into plasma membranes are mutually interdependent.

Tu-Pos416

EFFECT OF ELEVATED GLUCOSE AND SORBINIL ON THE [NA+,K+]-ATPASE ACTIVITY IN ISOLATED RAT AND HUMAN ERYTHROCYTES

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Derangements in the [Na+,K+]-ATPase activity within certain tissues of diabetic individuals has been implicated as the basis for some of the clinically and pathologically observed diabetic complications. However, the biochemical mechanisms responsible have not been fully elucidated. Due to multiple limitations in ascertaining precise biochemical and molecular interactions in multicellular and compartmented tissue based models, and the inability to obtain significant quantities of human material for experimental study, we have investigated the feasibility of studying the regulation of [Na+,K+]-ATPase activity in isolated erythrocytes, an easily obtained homogeneous preparation with many advantages.

Whole blood withdrawn from humans and Sprague-Dawley rats into heparinized tubes had the buffy coat removed, and the cells were washed three times in a modified Krebs medium. Aliquots of the isolated red blood cells were incubated in tubes containing the appropriate medium, and placed within a shaking water bath at 37 C. [Na+,K+]-ATPase activity was calculated from the measured ouabain sensitive ^{86}Rb uptake.

Erythrocytes from nondiabetic humans or rats, incubated in medium with 20 mM glucose for 90 minutes, had decreased [Na+,K+]-ATPase activity compared with 5 mM glucose controls (72% ($p<.005$) and 67% ($p<.0005$), respectively). The substitution of sucrose for the additional glucose in the incubation medium eliminated the inhibition of ATPase activity, suggesting that the effects are not associated with osmolality changes. In further studies using rat erythrocytes the addition of 0.1 mM sorbinil to the incubation medium containing 20 mM glucose completely reversed inhibition of ATPase activity ($p<.01$). Addition of 5 μM myo-inositol to the 20 mM glucose medium also independently reversed the effect of the glucose induced inhibition ($p<.01$). Other metabolic substrates of the erythrocyte, such as fructose and galactose, have similar downregulatory effects on the [Na+,K+]-ATPase activity, which are also inhibited by sorbinil.

We thus present evidence that the effect of in vitro high glucose levels on the erythrocyte [Na+,K+]-ATPase can be reversed by an aldose reductase inhibitor. The erythrocyte may be important as a model for the biochemical regulation of [Na+,K+]-ATPase in diabetes.

Supported in part by ADA, AHA, and HHMI

Tu-Pos417

INTERACTION OF MELITTIN WITH THE NA/K ATPASE: EVIDENCE FOR A MELITTIN-INDUCED CONFORMATIONAL CHANGE.

John Cuppoletti[a] and Alan J. Abbott[b]. Depts of [a]Physiology & Biophysics and [b]Pharmacology & Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati OH 45267.

The Na/K ATPase is inhibited by the bee venom polypeptide, melittin. KCl and NaCl protect the enzyme from melittin inhibition. Analysis of the K^+ and Na^+ protection against melittin inhibition suggested a kinetic model which was consistent with slowly reversible melittin binding, and mutually exclusive binding of melittin with K^+, and Na^+. Accordingly, in the absence of salt, the K_I for melittin inhibition = 1.2 uM, and the protection by KCl occurs with a $K_{A,KCl}$ = 0.6 mM. The protection by NaCl occurs with a $K_{A,NaCl}$ = 15 mM. Melittin inhibition of enzyme activity is due to direct interactions with the Na/K ATPase, as demonstrated by photolabeling with [^{125}I]azidosalicylyl melittin, which labeled the alpha subunit, but not the beta subunit of the Na/K ATPase. Melittin and KCl reduced the extent of labeling. In non-covalent binding studies using [^{125}I]azidosalicylyl melittin, 1.6 moles melittin binding sites were present per mole Na/K ATPase. Ligand-induced conformational changes of FITC-labeled Na/K ATPase were examined in the presence and absence of melittin. K^+ alone or melittin alone caused a fluorescence intensity quenching consistent with formation of an E_2 form of the enzyme. NaCl induced fluorescence changes did not occur when the enzyme was treated with melittin in the absence of K^+. However, when K^+ was present before the addition of melittin, NaCl induced fluorescence intensity increases were observed, which were dependent upon the concentration of K^+ in the pre-incubation mixture. The results of the labeling, kinetic, and conformational studies suggest a mechanism for interactions between ion pumps and (poly)peptides. It is known that proteins, including ankyrin, bind to the cytosolic regions of the Na/K ATPase, presumably regulating the distribution of the Na/K ATPase. The present studies suggest that proteins may be identified which regulate the function of the Na/K ATPase.

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Tu-Pos418

VOLTAGE DEPENDENCE OF ELECTRONEUTRAL Na/Na EXCHANGE VIA THE Na/K PUMP.

R.F. Rakowski, David C. Gadsby, and Paul De Weer. Marine Biological Laboratory, Woods Hole, MA 02543.

Transient pump currents recorded under Na/Na exchange conditions (e.g., Nakao & Gadsby, *Nature* **323**:628, 1986) imply that, although it is electroneutral, Na/Na exchange might nevertheless be voltage dependent. We tested this prediction by measuring dihydrodigitoxigenin (H_2DTG; 100 μM) -sensitive ^{22}Na efflux ($\Delta\Phi_{Na}$) at 21°C in voltage-clamped, internally dialyzed, squid giant axons. The K- and Cl-free (Cl replaced by isethionate and sulfamate) seawater included 0.2 μM TTX, 1 mM 3,4-DAP, 75 mM Ca, and 0-400 mM Na (Na replaced by N-methyl-D-glucamine). The K- and Cl-free internal fluid included (in mM): 50 Na, 15 Mg, 5 ATP, 5 ADP, 50 PPTEA, 2.5 BAPTA, 1.25 AP_5A, and 0.1 atractyloside. At -60 mV and 400 mM $[Na]_o$, $\Delta\Phi_{Na}$ was 15.1 ± 0.5 pmol cm^{-2} s^{-1} (n = 4). The simultaneously recorded holding current change (ΔI) was not significantly different from zero ($0.4 > p > 0.3$) as expected for electroneutral Na/Na exchange. Depolarization to 0 mV reversibly reduced $\Delta\Phi_{Na}$ to 9.0 ± 0.5 pmol cm^{-2} s^{-1} and resulted in the appearance of a small ΔI (0.028 ± 0.008 μA cm^{-2}) that, if it were produced by electrogenic 3Na/2Na exchange, could account for no more than 10% of the measured Na efflux. H_2DTG-insensitive ^{22}Na efflux was small (~5 pmol cm^{-2} s^{-1}) and independent of holding potential (V_H) or $[Na]_o$. Control tests confirmed that $\Delta\Phi_{Na}$ was sensitive to withdrawal of ATP, ADP, Mg_i, or Na_o. Measured over the range $-75 mV < V_H < +30 mV$, $\Delta\Phi_{Na}$ appeared saturated at $V_H < -45$ mV, declined for V_H positive to -45 mV, and was half-maximal near 0 mV. At 0 mV, $\Delta\Phi_{Na}$ depended linearly on $[Na]_o$ but, at -30 and -60 mV, $\Delta\Phi_{Na}$ was a hyperbolic function of $[Na]_o$ with an apparent K_m of 261 ± 27 mM (n = 3) and 121 ± 22 mM (n = 5), respectively. This voltage dependence of the apparent K_m for Na_o suggests that external Na activates Na/Na exchange from within a high-field access channel (ion well). *[Supported by NIH grants NS-22979, HL-36783, NS-11223, and the Irma T. Hirschl Trust]*

Tu-Pos419

SYNTHESIS AND INTERACTIONS OF THE SULFHYDRYL REACTIVE ATP ANALOG 8-THIOCYANATO-ATP WITH NA,K-ATPASE AND KINASES.

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The synthesis of 8-thiocyano-ATP (CNS^8-ATP) is described. At 37 °C the ATP analogue inactivates Na,K-ATPase, hexokinase and pyruvate kinase. In all three cases inactivation can be prevented by the addition of ATP, thus suggesting that CNS^8-ATP is recognized within the ATP binding site of these enzymes. Incubation of the inactivated enzymes with dithiothreitol restores the catalytic activities. Thus it is likely that in these enzymes a mixed disulfide (E-S-S^8-ATP) is formed between a sulfhydryl group in the ATP binding site (E-SH) and the ATP analogue:

$$\text{E-SH} + \text{CNS}^8\text{-ATP} \underset{K_D}{\rightleftharpoons} [\text{E-SH} \cdot \text{CNS}^8\text{-ATP}] \xrightarrow[k_2]{} \text{E-S-S}^8\text{-ATP} + \text{HCN}$$

From the pseudo first order inactivation kinetics a K_D = 2.7 μM with $k_2 = 0.142 \cdot min^{-1}$ is calculated for the hexokinase and a K_D = 40 μM with $k_2 = 0.347 \cdot min^{-1}$ is calculated for the pyruvate kinase interactions with the ATP analogue. At 4 °C Na,K-ATPase recognizes CNS^8-ATP with a K_D = 8.3 μM. At 37 °C the enzyme becomes inactivated by the ATP analogue in a biphasic manner. Inactivation results in the incorporation of [α-^{32}P] 8-CNS^8-ATP into the catalytic α-subunit of the enzyme. Limited tryptic digestion in the presence of 150 mM KCl results in the formation of a radioactive peptide of Mr = 56,000, known to bear the purine binding domain of Na,K-ATPase. The results described in this article indicate that CNS^8-ATP is a sulfhydryl reactive ATP analogue, and suggest that this new ATP analogue may be a useful tool for structure/function studies of ATP-binding enzymes. The isolation of a [2-^{3}H] CNS^8ATP labelled peptide from the ATP binding site of Na,K-ATPase bearing the sulfhydryl group(s) involved in ATP recognition is in progress.

Tu-Pos420

INVESTIGATION OF THE LATERAL AND ROTATIONAL MOTIONS OF THE CARBOHYDRATE CHAINS OF THE BETA-SUBUNIT OF Na,K-ATPase. E. Amler, H. Malak[1], A. Abbott, J. Lakowicz[1] and W.J. Ball, Jr. Dept. Physiology & Cell Biophysics, Univ. Cincinnati, Cinti., OH 45267-0575 and [1]Center for Fluorescence Spectroscopy, Dept. Biological Chemistry, Univ. Maryland School of Medicine, Baltimore, MD.

The Na,K-ATPase or Na-pump consists of at least two subunits, the catalytic α- and glycoprotein β-subunit. All ligand binding sites and catalytic functions have been ascribed to the larger α-subunit while the function of the smaller β remains unknown. However, the three carbohydrate (CH) chains which reside on the extracellular portion of the β have been hypothesized to be involved in enzyme association and insertion into the membrane and cell-cell interactions. An analysis of the lifetime of the excited state of lucifer yellow coupled to the CHs of β revealed two components in contrast to the one-component decay of lucifer yellow in buffer. Monitoring the nanosecond anisotropy decay, we found the existence of a relatively fast rotational motion of carbohydrates (rotational correlation time τ_c=1.5 ns) which is influenced modestly by Mg^{2+}. Analysis of the distance distribution as obtained from Forster energy transfer experiments (anthroyl ouabain as a donor, D, and lucifer yellow as an acceptor, A> using a static D-A distribution model vs. a diffusional model, suggested a surprisingly low diffusion coefficient for the carbohydrate chains (D <10^{-8} cm^2/S). Consequently, the lateral motion of the CH's of the Na,K-ATPase appear to be under substantial restriction. (Supported by NIH grant HL-32214 (WJB) and The Ohio AHA Fellowship SW-84-36-F (AA)).

Tu-Pos421

PHOTOAFFINITY LABELLING OF THE NUCLEOTIDE BINDING SITE OF NA,K-ATPASE USING 2-AZIDO-ATP. Chinh M. Tran, Edward E. Huston, and Robert A. Farley. Dept. of Physiology & Biophysics, University of Southern California School of Medicine, Los Angeles, CA 90033.

The adenine nucleotide analogs 8-azido-ATP and 2-azido-ATP have been widely used to identify amino acids within nucleotide binding sites of different proteins. Because both of these molecules bind to proteins in the anti-conformation, the presence of the photoactivated azide moiety on opposite sides of the purine ring provides the potential to identify amino acids that are located in different regions within ATP binding sites. We have previously shown that 8-azido-ATP labels the α subunit of Na,K-ATPase between amino acids 470-495. In the work reported here, 2-azido-ATP has been used to probe the ATP binding site of Na,K-ATPase with the goal of identifying additional amino acids within the ATP binding site. 2-azido-ATP was synthesized and was observed to irreversibly inhibit the hydrolysis of both ATP and p-nitrophenyl-phosphate by Na,K-ATPase after illumination with UV light. 50% inhibition of both activities was obtained using 5-15 μM 2-azido-ATP. Inhibition by 2-azido-ATP was prevented when ATP was included in the photolysis buffer. Protection was complete when the ratio of ATP/2-azido-ATP was greater than 40, up to 200 μM ATP. 2-azido-ATP competitively blocks the high affinity binding of ATP (Kd = 0.5 μM) to Na,K-ATPase in the absence of illumination with a Ki of 20 μM. [α-32P]-2-azido-ATP was synthesized and was observed to covalently label the α subunit of Na,K-ATPase. Trypsin digestion of the labeled protein released radiolabeled peptides to the supernatant after centrifugation, and the amino acid composition and sequence determination of these peptides is in progress. (Supported by GM28673, HL39295, AHA-GLAA).

Tu-Pos422

NIR Spectroscopy of the Kinetics of the Na-K ATPase-ATP Reaction

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Previous studies have shown the utility of NIR spectroscopy to identify and quantify aqueous mixtures of ATP,ADP, and H_2PO_4-HPO_4.[1] In conjunction with these results, the kinetics of the hydrolysis of ATP by Na-K ATPase have been monitored. The peak wavelength of ATP occurs at 1190 nm. Interestingly, upon addition of the enzyme, the peak position shifts to 1110 nm. Steady-state zeroth order rates can be readily followed at the peak wavelength of ATP absorption at 1110 nm. The origin of the shift as well as the kinetic results will be discussed in detail.

1. A. Mermelstein, et, al BJ vol 57, p54a (1990)

Tu-Pos422A

(Na^++K^+)-ATPase: ORIENTATIONS OF THE PHOSPHORYLATION SITE AND THE NUCLEOTIDE BINDING DOMAIN ON THE TRYPTIC FRAGMENTS OF THE α-SUBUNIT. N. Zolotarjova, W.-H. Huang, and A. Askari. Dept. of Pharmacology, Medical College of Ohio, Toledo, OH 43699-0008.

Exposure of the kidney enzyme to trypsin in the presence of K^+ cleaves the α-subunit between Arg-438 and Ala-439. While ATPase activity is abolished, E_1-E_2 transitions are normal in the cleaved enzyme; and Asp-369 of the N-terminal 48 kDa fragment, like that of the native α, is phosphorylated by ATP in a Na^+-dependent and K^+-inhibited manner. Inactivation, therefore, has been ascribed to reduction of ATP affinity at the high-affinity catalytic site due to the separation of Asp-369 from the nucleotide binding domain of the 64 kDa C-terminal fragment. Because of the paucity of data in support of this hypothesis, the present studies were done. Experiments on the Na^+-dependent phosphorylations of α and the 48 kDa peptide by ATP showed that $K_{0.5}$ of ATP was about the same (less than 1 μM) for both reactions. Fluorescein isothiocyanate (FITC) is known to react with Lys-501, or Lys-480, or Lys-766, and prevent the phosphorylation of α by ATP. When the FITC-labeled enzyme was exposed to K^++trypsin, the resulting 48 kDa peptide was not phosphorylated by ATP, showing that FITC that has reacted with the lysines of the 64 kDa fragment prevents phosphorylation of Asp-369 on the 48 kDa fragment. These findings clearly indicate that the juxtaposition of the phosphorylation site with the high-affinity nucleotide binding site or the FITC binding site is not disturbed by the trypsin split. The cause of inactivation resulting from this split remains to be determined. (Supported by NIH grant HL-36573).

Tu-Pos422B

(Na^++K^+)-ATPase: STUDIES ON THE IDENTIFICATION OF THE DIMERIZING DOMAIN OF THE α-SUBUNIT. M. Ganjeizadeh, W.-H. Huang and A. Askari. Dept. of Pharmacology, Medical College of Ohio, Toledo, OH 43699-0008.

We have shown before (JBC, 258, 9878-9885, 1983) that in the presence of copper-phenanthroline (CuP), phosphorylation of the enzyme induces the cross-linking of two α-subunits through disulfide bonds, and that this phenomenon is indicative of α,α-interactions of the membrane-bound enzyme. To determine which segments of the α-subunit are involved in the intersubunit contact between these sulfhydryl groups, we have subjected the purified kidney enzyme to controlled proteolysis and studied the phosphorylation-induced cross-linkings of the α and its resulting fragments. Chymotryptic cleavage in the presence of Na^+ yields an 83 kDa peptide (Ala-267 to C-terminus) that is phosphorylated on Asp-369. When a partially cleaved enzyme was phosphorylated and exposed to CuP, the induced cross-linked products were the α,α-dimer, the α,83-dimer, and the 83,83-dimer. If the chymotryptic cleavage was nearly complete, the only phosphorylation-induced cross-linked product was the 83,83-dimer. Tryptic cleavage in the presence of K^+ yields a 48 kDa peptide (N-terminus to Arg-438) that is phosphorylated on Asp-369, and a 64 kDa peptide (Ala-439 to C-terminus). When the partially trypsinized enzyme was phosphorylated and exposed to CuP, the induced cross-linked products were the α,α-dimer and one that was most likely the α,64-dimer. There was no evidence for the formation of a cross-linked 48,48-dimer involving the phosphorylated 48 kDa fragment. These experiments suggest that enzyme-enzyme interaction involves contact between segments of the α-subunit that are on the C-terminal side of Ala-439. (Supported by NIH grant HL-36573).

Tu-Pos423

EFFECT OF CHARGED RESIDUES ON THE BIOPHYSICAL PROPERTIES OF SIGNAL PEPTIDES. Jeffrey D. Jones, Sarah J. Stradley, Kim Khuan Ng and Lila M. Gierasch; Departments of Pharmacology and Biochemistry, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Tx 75235-9041.

Incorporation of charged residues in the hydrophobic core region of the E. coli LamB signal sequence has been shown to decrease in vivo secretion efficiency. The magnitude of the inhibition is dependent on both the nature and position of the charged residue. Arginine at position 17 inhibits more effectively than aspartate, but aspartate at the 13 position is more effective than either residue at position 17 (Stader et al. J. Biol. Chem. 261, 15075, 1986). We have compared secondary structure and lipid interactive properties of LamB mutants incorporating either arginine or aspartate in the 13 or 17 positions with the properties of the wild type signal peptide. All peptides studied demonstrate a high propensity for helical conformation in membrane mimetic environments by circular dichroism. The aspartate mutant peptides A13D and G17D exhibit decreased binding affinity for 1-palmitoyl-2 oleoylphosphatidylethanolamine(POPE)/POPG 65/35% monolayers relative to the wild type peptide. Prelminary experiments indicate that the charged mutant peptides show an ability to insert and perturb model lipid systems similar to that of the wild type peptide under conditions in which complete binding is obtained. The affinity and detailed mode of binding to lipid vesicles are being explored through the use of tryptophan-containing analogs of the charged mutant peptides. These physical studies will elucidate more fully the nature of the in vivo export defect manifested by these mutant signal sequences.

Supported by grants from the NIH (GM34962 to LMG, 5-F32-GM13341-02 to JDJ) and the NSF (DCB8896144 to LMG).

Tu-Pos424

DOES THE EXOCYTIC-SENSITVE PHOSPHOGLYCOPROTEIN, PARAFUSIN, UNDERGO AUTOPHOSPHORYLATION? A.P. Andersen and B. H. Satir Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, N.Y. 10461

Parafusin, an evolutionary conserved protein, has been hypothesized to be involved in one of the regulatory steps in membrane fusion in exocytosis in *Paramecium*; it dephosphorylates upon stimulation (Gilligan and Satir, J. Biol. Chem. 257, 13903, 1982). The phosphorylated form is cytosolic and parafusin has been isolated from the high speed supernatant (S2) from *Paramecium*. *Paramecium* parafusin has been reported to have two sites of phosphorylation: serine phosphate and glucose-phosphate-mannose O-linked to serine (Satir et al., J. Cell Biol. 111, 901, 1990). Parafusin enriched from *Paramecium* S2 was used to test for auto-phosphorylation in HEPES buffer, pH 7.3 in the presence of [γ^{32}P]-ATP at room temperature. Phosphorylation and the presence of parafusin is followed on autoradiogram of SDS PAGE and Western blots using the *Paramecium* anti-parafusin antibody. At pH 7.3 parafusin becomes phosphorylated. This appears to be independent of protein concentration, since in samples diluted 5, 20, 60 X phosphorylation still takes place. This reaction shows a pH dependence with an optimum at pH 7.5 and no phosphorylation occurs at pH 6.0-6.5. These results suggest that under these conditions parafusin is phosphorylated. This work was supported by NIH, GMS 32767 to BHS

Tu-Pos425

COMPARISON OF HELIX FORMATION AND STABILITY IN WILD TYPE AND MUTANT OmpA SIGNAL SEQUENCES. Bostjan Kobe, Josep Rizo, Martha D. Bruch and Lila M. Gierasch; Departments of Biochemistry and Pharmacology, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Tx 75235

Isolated signal peptides have a high tendency to form α-helices in membrane-mimetic environments and this ability seems to be required for their function (Jones et al. (1990), *J.Bioen.Biomemb.* **22**, 213-232). To further explore their conformational characteristics, two-dimensional proton NMR analysis of synthetic peptides corresponding to the signal sequences of OmpA protein of *E. coli* and several mutants has been carried out in 20 mol % trifluoroethanol in water, a solvent mixture which mimics the conformational distribution of these peptides in membrane-like environment. The functional peptides adopt an α-helical conformation which spans the major part of the signal sequence. A comparison with the isolated *E. coli* LamB signal peptide previously characterized by NMR (Bruch et al. (1989), *Biochemistry* **28**, 8554-8561) shows that the helix is longer in OmpA, but its most stable part in the hydrophobic core region is of the same length. The length, location and stabilty of the helix in the OmpA signal peptide and its mutants will be discussed in relation to their *in vivo* function.

Supported by NIH and NSF grants.

Tu-Pos426

EFFECTS OF $[Ca^{2+}]_{out}$ ON CONSTITUTIVE AND REGULATED COMPONENTS OF STRETCH-AUGMENTED NATRIURETIC PEPTIDE SECRETION BY RAT ATRIA. Ernest Page, Judy Upshaw-Earley, and Dorothy Hanck. The University of Chicago, Chicago, IL 60637.

In vitro rat atrial preparations stretched at 37°C by a distending pressure of 5.1 mm Hg in a modified Krebs-Henseleit solution as described by Page, *et al.* (Am. J. Physiol.: Cell Physiol., Nov. 1990) and rendered quiescent by 1 μM saxitoxin (STX) and 100 μM ryanodine were used to measure the effects of external Ca^{2+} concentration ($[Ca^{2+}]_{out}$, varied from 0.2 to 3.0 mM) on the rate of stretch-activated atrial natriuretic peptide (ANP) secretion. Under these conditions, the amount of immunoreactive ANP secreted (y, in pmoles/mg dry atrium) could be well described by a single rate constant k (in min^{-1}) according to the exponential relation $y = s\cdot(1-e^{-kt})$, where s = the maximal amount of ANP secreted (in pmoles/mg dry atrium) after a long time, t (in minutes). The rate of stretch-augmented secretion increased significantly as $[Ca^{2+}]_{out}$ was raised, but slowed progressively with time in a $[Ca^{2+}]_{out}$-dependent manner, e.g., at $[Ca^{2+}]_{out}$ = 0.2 mM, k (mean ± SE) was 0.015 ± 0.003 min^{-1} and the maximal amount of ANP secreted, s was 12 ± 4 pmoles/mg dry atrium (n = 6), while at $[Ca^{2+}]_{out}$ = 1.4 mM, k was 0.045 ± 0.005 min^{-1} and s was 2.9 ± 0.4 pmoles/mg dry atrium (n = 6). These results were unaffected by absence of ryanodine (in presence of 10 μM STX). Inhibition of ANP synthesis (and presumably of constitutive secretion) with cycloheximide (100 μg/ml) or anisomycin (26 μg/ml) did not significantly alter either the $[Ca^{2+}]_{out}$-dependent increase in k or the time- and $[Ca^{2+}]_{out}$-dependent decrease in s: at 1.4 mM $[Ca^{2+}]_{out}$ in presence of cycloheximide, k was 0.039 ± 0.009 min^{-1} and s was 2.9 ± 0.4 pmoles/mg dry atrium (n = 5); the corresponding values in anisomycin were k = 0.052 ± 0.004 min^{-1} and s = 3.2 ± 0.3 pmoles/mg dry atrium (n = 4). In unstretched atria, cycloheximide had no consistent effect on basal ANP secretion. Electron micrographs of stretched atria treated for 25 min with cycloheximide in 1.4 mM $[Ca^{2+}]_{out}$ showed that atrial myocytes had an apparently normal content of atrial granules. These observations suggest that, in pressure-distended (stretched) atria, the contribution of constitutive ANP secretion to total ANP secretion is very small, and that both positive modulation of stretch-activated secretion by $[Ca^{2+}]_{out}$ and time- and $[Ca^{2+}]_{out}$-dependent inactivation of this secretion reflect effects on the regulated component which consists of stretch-triggered translocation and exocytosis of atrial granules containing concentrated ANP. Supported by USPHS grants HL 10503 and HL 20592.

Tu-Pos427

ORGANELLE IMPORT PATHWAY OF APOCYTOCHROME *c*.

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Drosophila melanogaster apocytochrome *c*, made biosynthetically *in vitro* and purified to homogeneity, shows two phases (high- and low-affinity) in its uptake by mouse liver mitochondria in the presence of a cytosolic factor. These associations are reversible and the apoprotein equilibrates between the mitochondria and the medium, within several minutes at 28 °C or 4 °C. Nevertheless, it was shown that the associated apoprotein is in a protease-resistant compartment, indicating it is translocated across the outer membrane of the organelle, probably *via* a pore. That the apoprotein was also translocated across the inner membrane, was demonstrated by obtaining mitoplasts, *i.e.*, mitochondria with ruptured outer membranes, from organelles initially loaded with apocytochrome *c*. The mitoplasts still contained a large amount of apocytochrome *c*, even after treatment of the mitoplasts with protease. Reequilibration of the apocytochrome *c* between the mitoplasts and the external medium, however, could not be demonstrated at 28 °C nor at 4 °C. For mitochondria, internalized native protein can reequilibrate to the extent of about 95% with the external medium, and this material consists entirely of intact apocytochrome *c*. A large proportion of the remaining 5% was found to be holocytochrome *c*.

The heme-lyase reaction was not required in all these translocation phenomena since mutant apocytochromes *c*, in which the two cysteine residues were replaced by alanines or serines, behaved like the native apoprotein. Apocytochrome *c* constructs in which the apoproteins were truncated from the C-terminus to not more than residue 58, obtained by runoff translations, a mutant missing the segment between residues 35 and 66, and an apoprotein having a non-cytochrome sequence at residues 90 to 120, all behaved like the native apoprotein. In contrast, changing the 3 lysines in the N-terminal 13 residues to glutamine, glutamate and asparagine stopped the high-affinity uptake, similarly to what occurs with the presequences of other translocated protein precursors.

(Supported by NIH grant GM 12121)

Tu-Pos428

ENDOCYTIC RATE OF LIPOSOMES IN CV1 AND J774 CELLS CONTROLLED BY SPECIFIC PHOSPHOLIPID HEADGROUPS AND SURFACE CHARGE DENSITY, Kyung-Dall Lee, Keelung Hong, and Demetrios Papahadjopoulos, Cancer Research Institute, University of California, San Francisco, CA 94143.

CV1, an African Green Monkey Kidney cell line, and J774, a Murine Macrophage-like cell line, showed different rates of endocytosis toward liposomes of different surface properties. Negatively charged phospholipids incorporated into the bilayer of egg PC (phosphatidylcholine)/cholesterol (2/1) liposomes were recognized to different extents depending on the headgroup of the anionic phospholipid and its charge density in the liposome bilayer. In CV1 cells, liposomes containing 10% of PS (phosphatidylserine) or PG (phosphatidylglycerol) bound avidly and became endocytosed rapidly. Increasing the content of PS or PG beyond 10% did not show further enhancement in the rate of uptake. In contrast, liposomes containing 10% monosialoganglioside GM_1, PI (phosphatidylinositol), or PEG-PE (poly-ethylene glycol linked to phosphatidylethanolamine) did not bind or promote endocytosis. With J774 cells, PS, PG, and PI all showed dramatically enhanced endocytosis with increasing amounts incorporated in the liposome bilayer. At least 50% of PS, PG, or PI was needed to reach the level of uptake seen in CV1 cells. In all cases, the number of liposomes endocytosed at 37°C after 1 hour of incubation with cells was proportional to the number of liposomes bound to the same cells at 4°C for the same incubation period; the differences observed in the uptake of liposomes with different surface properties seemed to be modulated mostly by the binding step between the liposome surface and cell membrane components. Liposomes internalized by cells could be distinguished from those bound to the cell surface using a pH-sensitive fluorescent dye, Pyranine, encapsulated in the liposomes. The number of liposomes at the cell surface, L(s), reached a steady state within 5 minutes of incubation with the cells at 37°C. The number of liposomes inside the endocytic compartments, L(i), increased linearly with time, while L(s) stayed constant. From the relationship $dL(i)/dt = k_e L(s)$, a k_e (the endocytic rate constant) value of 0.06 was obtained for liposomes containing 50% PS both with CV1 and J774 cells. This value corresponds to approximately 300 liposomes endocytosed per minute per cell.

Tu-Pos429

INTERNALIZATION AND RELEASE OF TWO ISOMERS OF BENZOPYRENE BY DICTYTOSTELIUM DISCOIDEUM CELLS.

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The microorganism *Dictyostelium Discoideum* (Dd) has been used as an eukaryotic model to investigate the cell behaviour against Benzopyrene, the main carcinogenic compound of tobacco smoke. For this study, cells were first incubated for 1 hour with 10^{-5}M Benzo(a)-Pyrene [B(a)P] or 10^{-5}M Benzo(e)-Pyrene [B(e)P], two isomers respectively carcinogenic and non-carcinogenic for mammalian cells. Fluorescence measurements performed during starvation-induced development indicated that a much larger amount of the carcinogenic form [B(a)P], was released as compared to [B(e)P].
To precise the machinery used by Dd to release B(a)P, plasma membranes (PM) and coated vesicles (CV) were analyzed for their benzopyrene content. After cell homogeneization, CV were purified on sucrose-^2H_2O gradients. Plasma membranes were prepared from the same cell batches and purified on sucrose gradients. Large amount of both benzopyrene isomers was detected in PM by spectrofluorimetry. In contrast, only B(a)P seemed to be involved in the CV-mediated cellular traffic. Electrophoresis performed under non-denaturing conditions indicated that in CV and PM, B(a)P was clustered in a high molecular weight protein complex. Such clustering was not detected in the subcellular fractions of cells incubated with B(e)P. These results will be discussed taking into account two working hypothesis: Dd cells would get rid of the B(a)P carcinogenic compound either by increasing the CV-mediated traffic or by using a B(a)P-induced multidrug resistance mechanism.

This work was supported by a grant from Curie Institute.

Tu-Pos430

FLUORESCENCE EXCITATION RATIO PH MEASUREMENTS OF LYSOSOMAL PH USING LASER SCANNING CONFOCAL MICROSCOPY.

K.W. Dunn[1], F.R. Maxfield[1], J.E. Whitaker[2], R.P. Haugland[2] and R.P. Haugland[2], [1] Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, [2] Molecular Probes, Inc., Eugene, OR 97402 (Intro. by J.N. Myers).

Conjugates of pH sensitive, fluorescent dyes with dextran and transferrin were used to label endocytic organelles of NRK and CHO cells. These dyes, Cl-CNERF and DM-CNERF, have greater photostability and lower pKas than do comparable fluorescein derivatives making them better suited to confocal imaging of endocytic compartments of living cells. Using these fluorescent conjugates we have generated confocal images of living cells with brightly labelled lysosomes and other endocytic compartments. The fluorescence excitation spectra vary with pH in such a way that fluorescence with 514 nm excitation light is more highly pH dependent than that emitted with 488 nm excitation light. This property permits utilization of the primary emission lines of the argon laser used in laser scanning confocal microscopy to measure the pH of conjugate-containing compartments as the ratio of fluorescence emitted with 514 nm excitation to that with 488 nm excitation. The low pKas of these dyes permit the characterization of compartments whose pH is at or below the lower limit of fluorescein's utility, in this case lysosomes. Cells were incubated with fluorescent dextrans overnight and then in the absence of label for another hour to label lysosomes. To construct an *in situ* calibration curve, the intracellular compartments of fixed cells were equlibrated with a range of pH buffers and fluorescence ratios of labelled compartments measured. In living cells, a distribution of lysosomal pH values was found, including a previously undetected sub-population with pH < 4.8. These probes should permit the characterization of the 3-dimensional distribution and pH of acidic endocytic organelles via confocal microscopy.

Tu-Pos431

HETEROGENEITY IN ATP-DEPENDENT ACIDIFICATION IN ENDOCYTIC VESICLES FROM KIDNEY PROXIMAL TUBULE: MEASUREMENT OF pH IN INDIVIDUAL VESICLES BY IMAGE ANALYSIS. *L.-B. Shi, K. Fushimi, H.-R. Bae and A.S. Verkman. U.C.S.F.*

Measurement of membrane transport in suspensions of isolated membrane vesicles provides averaged information over heterogeneous vesicle populations. To examine regulatory mechanisms for ATP-dependent acidification, methodology was developed to measure pH in individual endocytic vesicles. Vesicles from rat kidney proximal tubule were labeled in vivo with FITC-dextran (J. Gen. Physiol. 93:885-902, 1989), immobilized on a poly-lysine coated coverglass and imaged at high magnification by a SIT camera. Endosome pH was determined from the integrated fluorescence intensity of individual labeled vesicles after background subtraction. Calibration studies with high K and nigericin showed nearly identical fluorescence vs. pH curves for different endosomes with a standard deviation for a single pH measurement in a single endosome of ~0.2 pH units. In response to addition of 1 mM MgATP in the presence of K and valinomycin, endosome pH decreased from 7.2 to a mean of 6.4 with a unimodal distribution with width at half-maximum of ~1 pH unit. The drop in endosome pH increased and the shape of the distribution changed when the time between FITC-dextran infusion and kidney removal was increased from 5 to 20 min. Differences in ATP-dependent acidification could not be attributed to heterogeneity in passive proton conductance. These results establish a direct method to measure pH in single endocytic vesicles and demonstrate remarkable heterogeneity in ATP-dependent acidification which was interpreted in terms of heterogeneity in the number and/or activity of proton pumps at serial stages of endocytosis.

Tu-Pos432

^{45}Ca UPTAKE BY MYXICOLA MITOCHONDRIA. N.F. Al-Baldawi and R.F. Abercrombie, Department of Physiology, Emory University, Atlanta, GA 30322.

We measured ^{45}Ca accumulation in an inulin-inaccessible compartment within the cytoplasm of the Myxicola giant neuron. Steady state accumulation, reached after 1 hour, approached, but did not reach, a maximum as the free calcium increased from 1 to 240 μM. Under our standard conditions of 40 μM free calcium, accumulation occurred without exogenous substrate; KCN inhibited this accumulation by 70%. In the presence of 1 mM ATP, calcium accumulation was inhibited 90% to 95% by dinitrophenol (DNP), carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP), or ruthenium red, suggesting that the mitochondria are responsible. Calcium accumulation was measured as a function of pH in buffers containing either 1 mM ATP, 5 mM succinate, or with no added substrate. In all solutions, calcium accumulation was nearly zero at pH 5 and pH 9, and was a bell-shaped function of pH between these values. Accumulation of the lipophilic cation tetraphenyl-phosphonium (TPP) into the inulin-inaccessible space was measured, with and without KCN, as a function of pH. TPP accumulation, a bell-shaped function of pH, was CN-sensitive. Comparison of the ^{45}Ca accumulation to the ^{14}C-TPP accumulation showed that calcium was lower than predicted assuming electrochemical equilibrium of these ions. The TPP measurements suggest that the effect of pH on calcium accumulation is partly the result of its effect on the membrane potential of the mitochondria.

Tu-Pos433

^{45}Ca UPTAKE BY MYXICOLA AXOPLASM: EFFECTS OF IP_3, GTP, AND CAFFEINE. J.E. Moore, N.F. Al-Baldawi, and R.F. Abercrombie, Department of Physiology, Emory University, Atlanta, GA 30322.

Axoplasm from the invertebrate giant neuron of Myxicola infundibulum contains organelles resembling smooth endoplasmic reticulum, as well as mitochondria and neurofilaments (Ortiz et al., BBA 814:13-22, 1984). In invertebrate axons, the smooth endoplasmic reticulum has been proposed to have a role in intracellular calcium regulation (Henkart et al., Science 202:1300-1303, 1978). Our aim was to determine whether agents such as IP_3, GTP, and caffeine, which are thought to have their effects on nonmitochondrial calcium stores, affect calcium accumulation in this preparation of neural cytoplasm. We found IP_3 caused a small but statistically significant ($P<0.05$) decrease in ^{45}Ca accumulation by samples of axoplasm incubated for 1 hr in buffer containing 5 μM Ca^{2+} at pH 6 or 8. IP_3 plus GTP had the same effect as IP3 alone and GTP alone had no significant effect. Total ^{45}Ca accumulation was a bell shaped function of pH with a maximum near pH 7. The absolute magnitude of the IP_3 plus GTP-sensitive component appeared to be independent of pH. At 5 μM Ca^{2+} and 1 hr of incubation, 10 mM caffeine had an insignificant effect on ^{45}Ca accumulation at pH 7 and caused a significant ($P<0.05$) increase in accumulation at pH 8.

Tu-Pos434

PROBING THE STRUCTURE OF THE RHODOPSIN BINDING DOMAIN OF ROD G PROTEIN

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The molecular basis of the interaction between the visual receptor, rhodopsin (R), and the rod outer segment GTP binding protein, G_t, was studied to elucidate the site(s) on the G_t α subunit (α_t) involved in high- affinity binding to light-activated rhodopsin. Synthetic peptides based on the amino acid sequence of portions of the molecule that interact with rhodopsin, including amino acids 311-329 and 340-350, can themselves bind to rhodopsin and thus behave as competitive inhibitors of R-G interaction. This blockade was assessed by measuring the ability of peptides to inhibit G_t stabilization of the active (metarhodopsin II) conformation of rhodopsin (Hamm et al., Sci. 241:832, 1988). In the absence of G_t these peptides directly bind to rhodopsin and stabilize the metarhodopsin II conformation, mimicking G_t binding. This property of the peptides allowed the determination of their structure when bound to rhodopsin, providing insight into the conformation of the region of the α subunit that interacts with rhodopsin.

The conformation of the synthetic peptide α_t-340–350 in solution and as it binds to rhodopsin was determined by 2D NMR and is reported by Dratz et al. (this volume). The three-dimensional structural information was combined with energy minimization calculations to construct a model of the peptide conformation. The structural features include a type II β turn from Cys^{347} to Phe^{350}. To test the model, peptide analogs that are predicted to break or stabilize a type II β turn were synthesized and tested. A d-Ala^{348}–substituted peptide, which is consistent with a type II β turn but would break other structures, was equipotent with the native peptide in stabilizing metarhodopsin II, whereas a Leu^{348}–substituted peptide, which should disrupt the β turn, was considerably less potent. The data support the model and underline the importance of a type II β turn in the rhodopsin-bound conformation of the peptide. (Supported by NIH EY06062, NSF DMB-8804861, Glaxo Cardiovascular Discovery Grant and MT Center for Excellence in Biotech)

Tu-Pos435

TWO SITE HIGH AFFINITY INTERACTION BETWEEN INHIBITORY AND CATALYTIC SUBUNITS OF ROD cGMP-PHOSPHODIESTERASE

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It is known that a positively charged region (24–46) of rod cGMP-phosphodiesterase (PDE) inhibitory gamma subunit (γPDE) interacts with α_t (1, 2). We now show that this region is also involved in interaction with the PDE catalytic subunits ($\alpha\beta$). A synthetic peptide corresponding to this site inhibited nearly 50% of trypsin-activated PDE (tPDE) (IC50 15μM) and competed with purified γPDE for inhibition of tPDE. Effects of this peptide on tPDE were similar to those produced by histone, which is known to activate basal PDE activity. The peptide also changed the composition of high molecular weight cross-linked products (MW>200 kDa) which appeared after treatment with phenylenedimaleimide (PDM). This domain, together with the C-terminus of γPDE (1, 3) could provide high affinity binding (Kd 10nM) of the molecule to $\alpha\beta$ PDE. Using anti-αPDE-1-15 anti-peptide antibodies (kindly provided by Dr. D. Takemoto) we showed that γPDE was cross-linked by PDM to both α and β PDE. Since Cys(68)-γPDE, which participates in cross-linking, is located between the γPDE C-terminus and γPDE-24-46, we conclude that a significant part of γPDE is in close proximity to $\alpha\beta$PDE.

These data are consistent with certain characteristics of PDE activation by rod G protein α subunit ($\alpha_t GTP\gamma S$). In experiments using tPDE and excess γPDE, $\alpha_t GTP\gamma S$ did not activate PDE until free γ-subunit was bound. We suggest that the different affinity of $\alpha_t GTP\gamma S$ for free γPDE and γ complexed to $\alpha\beta$ PDE is likely due to competition between α_t and $\alpha\beta$PDE for γ-24-46. These experiments exclude the formation of the complex $\alpha\beta$PDE-$\alpha_t GTP\gamma S$, however, they leave open the possibility of the existence of the intermediate complex $\alpha\beta$PDE-γPDE-$\alpha_t GTP\gamma S$.

1 Lipkin et al., (1988) FEBS Lett. 234:287-290.
2 Morrison et al., (1990) J. B. C. 264:11671-11681.
3 Brown and Stryer, (1989) PNAS 86:4922-4926.

Tu-Pos436

MAPPING FUNCTIONAL RESIDUES IN THE ALPHA SUBUNIT OF G_S

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The G proteins are a family of GTP-binding proteins that transduce extracellular signals to regulate many cellular processes, including activation and inhibition of adenylyl cyclase, mediated by G_S and G_i, respectively. To identify the residues that constitute the effector region of α_S, we produced chimeric proteins containing parts of α_S and α_{i2}. They were tested for their ability to activate adenylyl cyclase when expressed in COS-7 cells or in genetically α_S-deficient S49 lymphoma cells. The smallest linear stretch of α_S sequence required for a chimera to activate adenylyl cyclase was a 120-residue segment located within the COOH-terminal 40% of the protein. The fact that smaller α_S segments did not activate adenylyl cyclase suggested that discontinuous regions within the 120-residue segment were necessary for function. To identify these regions, small clusters of charged or polar residues within the 120-residue segment were changed to their homologs in α_{i2}. Seven of ten such changes had no effect on adenylyl cyclase activation. Of the three that resulted in loss of function, one maps close to the guanine nucleotide in the structure of *ras*. The other two are located on one side of the molecule, near the NH_2- and COOH-termini, and of these one maps onto an α helix, α2 of *ras*, which changes conformation upon activation of the protein by GTP (Pai *et al*, 1989, *Nature* 341:209-214).

Tu-Pos437

AUGMENTATION BY GAP OF THE RESPONSE OF THE OUABAIN-INSENSITIVE SODIUM EFFLUX IN BARNACLE MUSCLE FIBERS TO THE INJECTION OF Gpp(NH)p

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Previous work has shown that the injection of $GTPNa_2$ into barnacle muscle fibers stimulates the ouabain-insensitive Na efflux and that the response is markedly reduced by preinjecting PKI or pre-applying verapamil. The same is true if Gpp(NH)p is injected. Such results are in accord with the current view that Gs protein is involved in the activation of the membrane adenylate cyclase system, as well as voltage-dependent Ca^{2+} channels. Supporting evidence for this view also comes from experiments showing that the response to GTP or Gpp(NH)p injection is abolished by preinjecting Al^{3+}. This type of work has now been extended to include an investigation of the possibility that the response to guanine nucleotide injection might involve p21-ras protein rather than Gs or that both proteins are involved as modulators of adenylate cyclase activity. To this end, GAP (GTPase activating protein), supplied by Drs. Frank McCormick and Gideon Bollag of Cetus, which is a protein that promotes the association of GDP with p21, was injected into ouabain-poisoned fibers prior to Gpp(NH)p, while companion controls were injected with the buffer solution prior to the guanine nucleotide. The results obtained show that the injection of 0.5M-Gpp(NH)p following a 20 μM-GAP solution produced a stimulatory response of the ouabain-insensitive Na efflux, the magnitude of which averages 247$\pm$32% (n=11). This value is significantly greater than that of 163$\pm$22% (n=11) obtained with companion control fibers. Such results are consistent with the idea that GAP is a dual effector molecule for p21 ras and Gs proteins and that its overriding effect is that of facilitating the activation of Gs by Gpp(NH)p. Alternatively, if barnacle fibers do not possess p21 ras, then the present data suggest that GAP acts as a positive effector molecule for Gs protein.

Tu-Pos438

Alpha$_1$-Adrenoceptors Stimulate a Membrane-associated High-Affinity GTPase Activity in Rabbit Cardiac Myocytes.

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Activation of cardiac α_1-adrenoreceptors (α_1-ARs) is known to produce positive inotropy in the heart, although the underlying cellular events are poorly understood. At the level of the plasma membrane, α_1-ARs are thought functionally to interact with a pertussis toxin (PTX)-insensitive GTP-binding regulatory (G) protein. In order to provide direct evidence of G protein activation by α_1-ARs, we have examined biochemically the stimulation of GTPase activity by α_1 agonists in cardiac myocyte sarcolemma. Cardiac myocytes were isolated from both rabbit atria and ventricle by enzymatic dispersion, and a sarcolemmal fraction was prepared by discontinuous sucrose gradient centrifugation. Assays were performed at 30°C, and GTPase activity was measured as the enzymatic release of ^{32}Pi using (γ)^{32}P-GTP as the substrate. Hormone-stimulated activity was linear with incubation time up to 20 minutes. In the presence of 10 μM propranolol, the α_1-adrenergic agonists noradrenaline and methoxamine dose-dependently stimulated GTPase activity with EC_{50} values (5-10 μM and 200 μM, respectively) similar to those reported for the α_1-adrenergic modulation of the outward K^+ current, It, and positive inotropy. This α_1-AR-stimulated GTPase was found to be of high affinity (Km for GTP $\approx$ 150 nM) with a Vmax of 1-3 pmol Pi/min/mg protein (30-100% above control levels). However, the maximal level of α_1 stimulation was consistently less than that observed for carbachol-induced stimulation of muscarinic receptor-associated GTPase activity in this membrane fraction. Furthermore, pre-treatment of intact atrial and ventricular myocytes with 0.5 μg/ml PTX for 8-10 hrs did not block the α_1 stimulation of GTPase activity, in agreement with recent physiological and biochemical studies. In summary, these findings provide direct evidence that cardiac α_1-ARs couple to, and activate, a sarcolemmal G protein-associated GTPase activity. (Supported by the Alberta Heritage Foundation for Medical Research and the Heart and Stroke Foundation of Canada)

Tu-Pos439

PHOSPHORYLATION DEPENDENT REGULATION OF G-PROTEIN NUCLEOTIDE EXCHANGE BY A PEPTIDE DERIVED FROM MARCKS (MYRISTOYLATED ALANINE RICH C KINASE SUBSTRATE) PROTEIN.

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We have reacted GTPγS with methylisotoic anhydride to produce the methyl anthranoyl (MANT) ester of ribose on GTPγS. MANT-GTPγS undergoes a large (21-fold) fluorescence increase (at 480 nm) upon binding to G-protein (G_o,G_i), when it is excited via resonance energy transfer (excitation at 280 nm) from G-protein tryptophan residues and a 2.5 fold fluorescence increase with direct excitation at 370 nm. MANT-GTPγS binding to G-protein was inhibited by unlabeled GTPγS and by GDP suggesting that it is directed to the nucleotide binding site of the G-protein. The fluorescence increase in MANT-GTPγS has allowed us to determine the rate of its binding to G-protein, a process limited by the dissociation of GDP. MANT-GTPγS (100 nM) on-rate to G-protein (50 nM) was determined to be 0.006/sec at 25 °C, consistent with previously reported GDP off-rates.

A peptide (KKKKKRFSFKKSFKLSGFSFKKNKK) which contains the phosphorylation site domain of the MARCKS protein (provided by Dr. Perry J. Blackshear of Duke University) increased MANT-GTPγS on-rate 2.5, 4.0, and 7-fold at 30, 50 and 100 nM peptide, respectively. MARCKS-peptide facilitation of MANT-GTPγS exchange with G-protein was completely abolished by PKC dependent phosphorylation of the peptide.

Our results suggest that the MARCKS protein may function to activate G-protein within the cell in a manner which is regulated by protein kinase C dependent phosphorylation.

Tu-Pos440

TIME-RESOLVED MICROCALORIMETRIC STUDIES OF TRANSDUCIN DEACTIVATION.

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The subsecond[1] electrophysiological response of the retinal rod cell to a weak flash is mediated by a classic G-protein cascade: the receptor is rhodopsin (R), the G-protein is transducin (T) and the effector is the cGMP phosphodiesterase (PDE). Termination of this response requires endogenous GTP hydrolysis[2]. Yet steady-state measurements of the GTPase activity of transducin yielded *in-vitro* rates of a few GTP hydrolyzed per transducin per minute[3,4], implying a deactivation time of many seconds for TαGTP. But this is in fact the total duration of the entire *in-vitro* transducin cycle of which the active lifetime of TαGTP might be but a small fraction. We have used time-resolved microcalorimetry[5] to monitor in bovine rod outer segments the heat due to GTP hydrolysis by a transducin population that had been quickly activated by flash illumination of rhodopsin. At 22°C, the heat pulse observed lasts less than a second. This heat consists of the exothermic activation of transducin by R*-catalyzed GTP loading and the subsequent hydrolysis of GTP by Tα. The activation heat was directly measured using the non-hydrolyzable analog GTPγS and the hydrolysis heat was obtained by subtraction. The activation heat precedes the hydrolysis heat which lasts less than a second. These results indicate that endogenous GTP hydrolysis by transducin is fast enough to account for the termination kinetics of the physiological response. After each heat pulse, there is a refractory time of many seconds during which transducin seems unable to reload GTP. This "dead" time may explain the reported low steady-state *in-vitro* GTPase rates. Interaction of TαGTP with PDE or other yet-to-be-described membrane-associated proteins might be responsible for the fast rate of GTP hydrolysis reported here.

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Tu-Pos441

CONTINUOUS ASSAY FOR PHOSPHATE RELEASE BASED ON uV ABSORBANCE MEASUREMENTS AND ITS USE TO MEASURE GAP-ACTIVATED HYDROLYSIS OF GTP BOUND TO p21ras.

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In the pH range 6 - 8.5, 2-amino-6-mercapto-7-methyl-purine ribonucleoside (MESG) gives an increase in absorbance at 360 nm when it reacts quantitatively with phosphate, catalyzed by purine nucleoside phosphorylase. This reaction can be used to quantitate phosphate in solutions in the micromolar range, or to follow the kinetics of phosphate release from biological systems, such as ATPases and GTPases.

This assay system has been used to measure ras GAP (GTPase activating protein) activity and the rate of phosphate release on hydrolysis of the GTP complex of p21ras. At 30°C and rates up to the limits of manual mixing, the rate of phosphate release is the same as that for GDP formation, indicating that phosphate release is fast relative to the hydrolysis step and that there is very little bound phosphate in the form of a GDP.P_i complex. The assay provides a simple probe for the interaction of GAP with GTP complexes of ras, by measuring the resultant hydrolysis for wild type ras protein or measuring inhibition by mutants.

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Tu-Pos442

KINETICS OF MUSCARINIC AGONIST DEPENDENT ACTIVATION AND DEACTIVATION OF THE G-PROTEIN COUPLED K^+ CURRENT $I_{(ACh)}$ IN ATRIAL MYOCYTES.

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Activation of atrial muscarinic (M_2) receptors induces an inwardly rectifying potassium current, $I_{(ACh)}$, mediated by a guanine nucleotide binding protein, G_k. We have explored the kinetics of this receptor mediated G_k activation and deactivation in intact cells by examining the time course of $I_{(ACh)}$ following rapid application and washout of ACh in single atrial cells of the bullfrog (*Rana catesbeiana*) heart. Membrane currents were measured under voltage-clamp using the whole-cell patch clamp technique that also allowed intracellular application of various GTP analogs. Rapid extracellular application and removal of agonist was accomplished using a valve that allowed complete replacement of the superfusing solution within 15 ms (10%-90%, as measured by changes in K^+_o). For a wide range of ACh concentrations (10nM-1mM), the activation of $I_{(ACh)}$ followed a time course that could be fit with a single exponential. In cells dialysed with the hydrolysis-resistant GTP analog GMP-PNP to eliminate G_k deactivation by GTP hydrolysis and in the presence of GDPβS to eliminate endogenous GTP production, the agonist dependence of the rate of $I_{(ACh)}$ activation calculated as the inverse of the time constant, followed a simple dose-effect relationship with a $K_{0.5}$ of 120nM ACh and a Hill coefficient (n_H) of 1. The limiting rate R_{MAX} was 13.4 min^{-1} for 0.2mM GMP-PNP. It increased as a function of the trinucleotide concentration following a dose-effect relationship with $K_{0.5}$=0.60mM GMP-PNP, n_H=2.4 and R_{MAX}=198 min^{-1}. Similar results were obtained for the activation rates in the presence of intracellular GTP ($k_{0.5}$=158nM ACh, n_H=1) with the exception of R_{MAX} which was 642 min^{-1}. In the presence of intracellular GTP $I_{(ACh)}$ deactivation upon ACh washout also followed an exponential time course with a rate (calculated as the inverse of the time constant of deactivation) near 120 min^{-1}. These results indicate that the kinetics of receptor-dependent G_k activation can be described as a two-state system in which only the activation rate, presumably corresponding to the release of GDP from G_k, is influenced by the concentration of the agonist in a simple dose-dependent manner.

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Tu-Pos443

ELECTRIC-FIELD INDUCED AGGREGATION OF CELL SURFACE PROTEINS CAUSES RISE IN INTRACELLULAR FREE CALCIUM LEVELS

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Activation of mast cells and basophils by binding of ligands that cross-link and micro-aggregate cell surface receptors leads to a series of responses including a phosphoinositide cascade, elevation of free intracellular $[Ca^{2+}]_i$ levels, morphological changes in the cell plasma membrane and ultimately, exocytosis of granules containing histamines and other mediators of the allergic response. In rat basophilic leukemia (RBL) cells, a tumor mast cell line, stimulation by IgE-receptor cross-linking induces these responses. McCloskey, Liu and Poo (*J. Cell Bio.*. 99 778, 1984) and Ryan et al.(*Science* 239 1, 1988) have shown that applied electric fields redistribute cell surface proteins on RBL cells. In order to determine whether aggregation of cell surface proteins is sufficient to induce the response in these cells without extrinsic cross-linking, we have crowded cell surface proteins together by electrophoretic segregation and looked for second messenger $[Ca^{2+}]_i$ responses. We are using fluorescence imaging techniques with the calcium sensitive dye FURA-2 to study the effects of receptor aggregation induced by application of small DC electric fields (5–15V/cm). In preliminary experiments we have observed a rise in intracellular $[Ca^{2+}]_i$ levels within a few minutes after the field is turned on, while redistribution is still in progress. Whether this response is a direct result of aggregation or a some other consequence of electrophoresis and whether the entire sequence of responses induced by antigen stimulation can be stimulated by electrophoresis is subject to continued research.

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Tu-Pos444

THE EFFECTS OF CALCIUM INDUCED-CALCIUM RELEASE INHIBITORS ON CALCIUM OSCILLATIONS IN HeLa CELLS.

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It has been proposed that Ca++ oscillations can be interpreted in terms of models based on a calcium induced-calcium release (CICR) mechanism. Fura-2 experiments were thus undertaken to investigate the possible contribution of a CICR mechanism, to the histamine induced Ca++ oscillations in HeLa cells, using the CICR inhibitors: ryanodine, procaine and adenine. It was observed that all of these drugs abolishe the appearence of Ca++ spikes during the oscillatory process, without inhibiting the initial Ca++ release induced by histamine. This inhibitory effect was also observed in nominally Ca++-free medium, indicating that the action of these CICR inhibitors is mainly located at the level of internal Ca++ reservoirs. These observations suggest therefore the existence of a Ca++ release mechanism other than the initial Ca++ release triggered by IP_3. In addition, the contribution of a CICR mechanism was investigated by measuring the kinetic of intracellular Ca++ changes in response to an increased Ca++ influx. We observed that after the run down of the Ca++ oscillations initiated by histamine in the absence of extracellular Ca++, the perfusion with a histamine-free bathing medium containing 1.8 mM Ca++, initiated a biphasic rise in intracellular Ca++, with a seccond phase related to a IP_3 independent Ca++ release from intracellular pools. This biphasic Ca++ release was prevented or inhibited by adenine. These results suggest the presence of mechanisms for the periodic release of Ca++ from internal stores: namely a IP_3-induce Ca++ release and CICR mechanism. This work was supported by M.R.C

Tu-Pos445

ENDOCYTOSIS OF LIGAND-RECEPTOR COMPLEXES: A SIMPLE QUANTITATIVE ANALYSIS OF A NONLINEAR SYSTEM.

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Introduction of a protein ligand to cells results in endocytotic internalization of the ligand-receptor complex. Within the initial 10 to 15 minutes of exposure to the ligand, the concentration of the (radioactive) internalized ligand plotted against the integrated concentration of surface bound ligand follows a linear relationship (Opresko and Wiley, J. Biol. Chem. 262: 4116; 1987). Thereafter, this relationship becomes nonlinear. We wish to evaluate quantitatively the dynamics of endocytosis observed under nonlinear conditions, when measured are the surface-associated radioactivity (SR), the total internalized radioactivity (IR) and the radioactivity excreted by cells (ER). Our basic model is expressed by the following relationship among these quantities:

$$[IR](t) = k\int_0^t [SR](u)du - [ER](t)$$

where t denotes the time dependence and k the endocytotic rate constant which can be determined by linear least-square fitting. Also, we propose a model based on measured values of SR and IR only. A variant of this model includes two regimens of endocytosis described by two rate constants. Further refinement of this model encompasses the nonlinear effects of receptor recycling. Supported by CA 45312.

Tu-Pos446

SURFACE BINDING KINETICS OF A MONOCLONAL Fab FRAGMENT ON SUPPORTED PHOSPHOLIPID MONOLAYERS MEASURED BY TOTAL INTERNAL REFLECTION/ FLUORESCENCE PHOTOBLEACHING RECOVERY

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Total internal reflection / fluorescence photobleaching recovery (TIR/FPR) has been used to measure the association/dissociation kinetics of a fluorescein-labeled anti-dinitrophenyl monoclonal Fab fragment specifically bound to supported phospholipid monolayers composed of a mixture of dipalmitoylphosphatidylcholine and dinitrophenyl-conjugated phosphatidyl-ethanolamine. The fluorescence recovery curves were biexponential with recovery rates of 1.4 sec^{-1} (~50%) and 0.11 sec^{-1} (~30%). Comparison of these measured off-rates with the previously measured association constant (3 x 10^5 M^{-1}) for this model system suggests on-rates of 3 x 10^4 $M^{-1}s^{-1}$ and 4 x 10^5 $M^{-1}s^{-1}$. The recovery curves did not significantly depend on the solution concentration of Fab fragments. These two results indicate that the surface reaction was not diffusion-limited. The biexponential character of the measured recovery curves may suggest multiple bound states and/or a multi-step binding mechanism. This work was supported by NIH grant GM-37514 and NSF grant DCB-8552986.

Tu-Pos447

ADVANCES IN POLARIZED FLUORESCENCE DEPLETION MEASUREMENT OF CELL MEMBRANE PROTEIN ROTATION. B.G. Barisas, T.R. Londo, J.R. Herman, N.A. Rahman and D.A. Roess, Departments of Chemistry and Physiology, Colorado State University, Fort Collins, CO 80523

The laser microscopic method of polarized fluorescence depletion (PFD) has permitted the rotational dynamics of functional membrane proteins to be measured on single cells under physiological conditions. A new cuvet implementation of this method permits small volumes of dilute cell suspensions to be examined in their entirety. This increases signal-to-noise ratios and data acquisition rates to values comparable to those obtained with time-resolved phosphorescence anisotropy (TPA) but with up to a 1000-fold reduction in sample requirements. A general theory for PFD experiments has been developed which permits interpretation of the results of these cuvet experiments. The sensitivity of these methods has enabled new studies of the function of luteinizing hormone receptors and MHC class I antigen on a variety of cell types where only very low numbers of receptors are expressed per cell and where small numbers of primary-cultured cells are available. In addition, a new photomultiplier gating device and system timing strategy reduce the minimum observable rotational correlation times to < 1 μsec, a 10-fold improvement over previous systems. These speed improvements have been examined in studies of EITC-BSA rotation in glycerol solution. TPA measurements can also be performed in the same instrument. This permits direct comparison of PFD and TPA results obtained under otherwise identical conditions. Supported by NIH grants HD-23236 (DAR) and AI-21873 and AI-26621 (BGB).

Tu-Pos448

PHOTOPROXIMITY LABELING OF THE Fc_ϵ RECEPTOR COMPLEX ON 2H3-RBL CELLS. B.I. Meiklejohn, N.A. Rahman, D.A. Roess, and B.G. Barisas (Intro. by R.W. Woody), Departments of Chemistry and Physiology, Colorado State University, Fort Collins, CO 80523.

We present optimized experimental conditions whereby the photoactivatable probe 5-iodonapthyl azide (INA) covalently derivatizes plasma membrane proteins physically proximal to specific target proteins. Normally, the hydrophobic molecule INA inserts uniformly in the membrane bilayer and is inert toward 514 nm light. However, the compound can be activated at 514 nm through energy transfer from certain donor fluorochromes on antibodies bound to specific membrane proteins. This sensitized activation occurs at distances of up to 100Å to yield an active nitrene which reacts with nearby membrane proteins. These derivatized proteins can be isolated and identified by gel electrophoresis and autoradiography. We have used this method to examine Type I Fc_ϵ receptors on rat 2H3 basophilic leukemia cells incubated with IgE in the presence and absence of a second, monoclonal anti-rat IgE antibody that efficiently crosslinks IgE-occupied Fc_ϵ receptors. When Fc_ϵ receptors are occupied by IgE alone, INA derivatizes four peptides with molecular weights of 53K, 38K, 34K and 29K. The 53K, 38K, and 34K peptides correspond well to molecular weights reported for the α-chain, the α-subunit and the β-chain of the IgE receptor. Following antibody-induced crosslinking, three additional peptides with molecular weights of 60K, 54K and 43K become associated with the IgE receptor. These studies suggest that additional membrane proteins are recruited into a complex containing IgE receptors following crosslinking and are consistent with polarized fluorescence depletion measurements of Fc_ϵ receptor rotational diffusion in which large, rotational immobile complexes are formed following receptor crosslinking. Supported by NIH grants AI-21873 and AI-26621 to B.G.B.

Tu-Pos449

KINETICS OF THE FORMATION OF Fc_ϵRI-DIMERS ON MAST CELLS AS CAUSED BY THE BINDING OF MONOCLONAL RECEPTOR SPECIFIC ANTIBODIES.

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The rates of association and dissociation of monoclonal Fc_ϵRI-specific antibodies (designated as J17 and F4) to and from this receptor were measured on live rat muscosal type mast cells (line RBL-2H3) at three different temperatures (25 °C, 15 °C and 4 °C). These IgG-class mAbs were shown earlier to dimerize the Fc_ϵRI (1,2). These studies revealed that the interactions between the respective Fab-fragments and the Fc_ϵRI are best fitted by a two-step reaction involving a conformational transition of the receptor-ligand complex (l->h). Analysis of the kinetics of interaction between these intact mAbs (IgG class) and the Fc_ϵRIs requires therefore also consideration of the l->h transition of the receptor-ligand complex. This was done by assuming involvement of the following species of Fc_ϵRI-dimers: an all-l-state dimer (D_{ll}), a hybrid (D_{lh}) with one receptor-ligand complex in the low and the other in the h-state and an all-h-state dimer (D_{hh}). By fitting the experimental data to this model a selfconsistent set of rate constants were derived. It turns out that the dimerization step is driven by a cooperative effect. While the formation of the hybrid D_{lh} encounters some strain und is therefore less favourable, the $D_{ll} \rightarrow D_{hh}$ transition is favoured by significant enthalpic contributions. At equilibrium a significant percentage of dimers is in the conformation D_{hh} which dissociates on a time scale larger than 10^5 sec.

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Tu-Pos450

COMBINED IMAGING OF $[Ca^{2+}]_i$ AND MEMBRANE POTENTIAL IN INDIVIDUAL TUMOR MAST CELLS DURING IgE RECEPTOR CROSSLINKING

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Antigen-induced depolarization and exocytosis in a mast cell line (RBL-2H3) requires influx of extracellular Ca^{2+} under normal physiological conditions. Digital imaging microscopy of fura-2 fluorescence has previously shown that both oscillations and a sustained rise in intracellular free ionized calcium ($[Ca^{2+}]_i$) occur in response to surface IgE receptor crosslinking by antigen (Millard et al. 1989. *J. Biol. Chem.* 264:19730). Studies using cell suspensions have shown that the accompanying depolarization of the plasma membrane is probably due to influx of extracellular Ca^{2+}. We have used imaging techniques to measure bis-(1,3-dibutylbarbiturate)trimethine oxonol (bis-oxonol) and fura-2 fluorescence simultaneously in individual cells to investigate the relationship between Ca^{2+} influx and the changes in $[Ca^{2+}]_i$ that lead ultimately to secretion. A rise in bis-oxonol fluorescence, indicating plasma membrane depolarization, was observed in fewer than half of the cells in response to concentrations of antigen that elicit a marked $[Ca^{2+}]_i$ response from >80% of the cells. Depolarization was preceded by a latent period of variable duration and usually followed the initial release of Ca^{2+} from intracellular stores by more than 10 sec. The $T_{1/2}$ of the increase in bis-oxonol fluorescence was 20-60 sec, which is similar to that measured in cell suspensions. Oscillations in membrane potential following antigen-stimulation occured in fewer than 1% of the cells, while greater than 50% of the cells showed oscillations in $[Ca^{2+}]_i$. However, when oscillations in membrane potential did occur, they were superimposed on a sustained depolarization and had a period that ranged from 10-20 sec, which is similar to that of oscillations in $[Ca^{2+}]_i$. The effects of fura-2 loading and the ionic requirements for the bis-oxonol response will be described.

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Tu-Pos451

Photolytic release of $InsP_3$ evokes fast rises of $[Ca]_i$ and potassium conductance in guinea-pig hepatocytes.

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Guinea-pig hepatocytes respond to Ca-mobilising hormones with a rise of membrane potassium conductance (g_K) activated by Ca-ions released from internal stores by $InsP_3$. Following a pulse of $InsP_3$ released by flash photolysis of caged $InsP_3$ (halftime 3ms) there is a delay of upto 1s in activation of g_K at low concentrations of $InsP_3$ (0.25 μM). Experiments with photolytically released Ca show that this delay is not due to slow activation of g_K by Ca, and could be reduced to 50 ms by raising cell free [Ca] prior to photolytic $InsP_3$ release (Ogden, Capiod, Walker & Trentham, 1990) suggesting that it represents time taken for [Ca] at the membrane to rise from rest to the level of 0.3 μM which activates g_K. To investigate the initial rate of $InsP_3$-evoked Ca release the time course of whole cell free [Ca] and g_K increase were measured simultaneously in the same cell with Fluo-3 fluorescence and whole cell patch clamp recording. Following photolytic release of low $[InsP_3]$ the fluorescence signal rose before g_K. At high $[InsP_3]$ (100 μM) both fluorescence and conductance rose after 25 ms. g_K rose steeply to a peak in 0.2 s. The results suggest that most of the delay in g_K at low $[InsP_3]$ can be accounted for by a slowed rise of Ca at the plasma membrane compared with the cell interior, and a steep dependence of g_K on [Ca]. At high $[InsP_3]$ the short delay in the Fluo signal of 25 ms after photolysis is consistent with a direct action of $InsP_3$ on receptors to produce Ca efflux from stores.

Ogden,D.C., Capiod,T., Walker,J.W. & Trentham,D.R. (1990) Journal of Physiology 422, 585-602.

Tu-Pos452

EVIDENCE THAT THE INHIBITION OF CALCIUM INFLUX IN RAT BASOPHILIC LEUKEMIA CELL CYTOPLASTS IS DUE TO THE ABSENCE OF INTRACELLULAR CALCIUM STORES. F. Charles Mohr and Sheryl K. Dunston, Department of Veterinary Pathology, University of California, Davis, CA 95616.

Rat basophilic leukemia (RBL) cells, a tumor analog for mast cells, secrete allergic mediators when receptors for immunoglobulin E (IgE) are aggregated by antigen. Antigen-mediated secretion in RBL cells depends on an increase in the cytoplasmic concentration of free ionized calcium (Ca^{2+}). This increase in cytoplasmic Ca^{2+} comes from both release of Ca^{2+} from intracellular stores and influx of external Ca^{2+} but the relationship between these two processes is not clear. It has been shown that antigen-stimulated RBL cell cytoplasts (cells lacking a nucleus and internal organelles) fail to increase their cytoplasmic Ca^{2+} concentration when stimulated, indicating that there is an uncoupling of IgE receptor aggregation and opening of plasma membrane Ca^{2+} permeability pathways. Since cytoplasts do not have intracellular Ca^{2+} stores, the lack of an increase in cytoplasmic Ca^{2+} during stimulation must be due to the inhibition of external Ca^{2+} influx. In an attempt to define the mechanism responsible for this inhibition, we have examined the early morphological and biochemical changes in cell stimulation. The measurement of cytoplasmic Ca^{2+} in individual resting and stimulated cytoplasts by ratio image analysis of fura-2 confirms that cytoplasts do not respond to antigen but do respond to ionomycin. Binding of fluorescein-IgE forms a typical ring form staining pattern, and addition of antigen causes the patching of IgE receptors, indicating that receptor aggregation is intact in RBL cytoplasts. Measuring the membrane potential of suspensions of cytoplasts with bis-oxonol demonstrates that the RBL cell cytoplast membrane is not depolarized, a phenomenon which is known to inhibit antigen-stimulated Ca^{2+} influx in RBL cells. Cholera toxin or mastoparan activation of G-proteins does not overcome the inhibition of Ca^{2+} influx into antigen-stimulated cytoplasts, thus ruling out a potential defect in G-protein effector coupling. Taken together, these findings suggest that the lack of antigen-stimulated Ca^{2+} influx into RBL cell cytoplasts is probably not due to a problem in any plasma membrane-located mechanism associated with the early stages of signal transduction. If antigen-stimulated Ca^{2+} influx is mediated by a rise in the intracellular Ca^{2+} concentration, the defect might be related to the absence of internal Ca^{2+}-releasing stores. Other possibilities, such as an effect of altered cytoskeletal elements on the inhibition of antigen-stimulated Ca^{2+} influx in cytoplasts, should be considered. This work is supported by BRS grants 89-13 and 90-16.

Tu-Pos453

INITIATION KINETICS OF CALCIUM TRANSIENTS: A SIMPLE SINGLE CASCADE MODEL FOR STIMULUS-RESPONSE LAG TIMES. Mȳ G. Mahoney, Linda L. Slakey, and David J. Gross. Program in Molecular and Cellular Biology and Department of Biochemistry, University of Massachusetts, Amherst, MA, 01003.

Interactions between extracellular adenine nucleotides and their respective purinergic receptors on porcine and rat aortic smooth muscle cells (ASMC) result in dynamic changes in intracellular calcium. Using a digital fluorescence imaging system, we measure both spatial and temporal changes in intracellular free calcium in individual adherent cells which are loaded with the calcium-sensitive indicator Fura-2. We observe in porcine ASMC a rapid initial transient rise followed by periodic oscillations in cytosolic $[Ca^{+2}]$ in response to an extracellularly applied dose of ATP. The initial transient is mediated by ATP through a high affinity receptor; the EC_{50} for producing the initial transient is 0.08μM. At receptor saturating doses of ATP, all cells respond rapidly (within 3 second resolution of our experiments) while at lower doses, of the order of 0.08μM and below, individual cells respond with variable lag times. Initial transients are quite rapid, rising from basal $[Ca^{+2}]_i$ to peak $[Ca^{+2}]_i$ in a few seconds. The initial transients in rat aortic cells follow a similar pattern, except the rise of the transient is slowed at low ATP doses.

In our model, the kinetics of ligand binding to cell surface receptor drive the catalytic production of activated G proteins, which in turn produce activated phospholipase C (PLC). PLC generates IP_3 which cooperatively (n=3) releases Ca^{+2} from IP_3-sensitive stores. All steps in the cascade are assumed to 1) inactivate slowly in comparison with the time necessary to develop the $[Ca^{+2}]_i$ response, 2) have molecular complexes which are formed rapidly, and 3) consume only a small portion of any activatable species. The model predicts that the cell acts as a temporal integrator and that the initiation of a rise in $[Ca^{+2}]_i$ occurs explosively after a lag period which is a function of ligand concentration, receptor density, and concentration of cascade signaling molecules.

Supported by NIH HL 31854 and NSF DMB-8803826

Tu-Pos454

EVIDENCE FOR TWO DOMAINS IN THE CYTOPLASMIC PORTION OF THE ASPARTATE RECEPTOR FROM *ESCHERICHIA COLI*. David G. Long* and Robert M. Weis†*. †Department of Chemistry, *Molecular and Cellular Biology Program; University of Massachusetts, Amherst, MA 01003.

The aspartate receptor is composed of a 60 kDa polypeptide, which has two transmembrane sequences and which is organized into a periplasmic ligand-binding domain and a cytoplasmic portion responsible for signaling and adaptation. A cloned c-terminal fragment of the receptor (31 kDa) has been purified and studied by differential scanning calorimetry (DSC) and circular dichroism spectroscopy. Both DSC and the temperature-dependence of the ellipticity at 222 nm indicate that the fragment is composed of at least two cooperative units. The presence of more than one cooperative unit observed in protein unfolding can be taken as evidence for the presence of more than one structural domain. The fragments from two missense mutants, which are phenotypically 'locked' into either smooth or tumbling swimming states, thus have distinguishable patterns of domain-domain interaction and domain stability. The implications of two structural domains on receptor signaling function will be discussed.

This work was supported by the grant GM-42636 from the NIH.

Tu-Pos455

FLUORESCENCE ENERGY TRANSFER MEASUREMENTS TO ANALYZE CD4 CONFORMATION ON PERIPHERAL BLOOD T CELLS. G. Szabo Jr., S. Pine, J. Weaver and A. Aszalos. CDER, Food and Drug Administration, Washington, D.C.

The helper/inducer T cell marker CD4, also the binding site for HIV gp120, is believed to become associated with the TcR/CD3 complex upon T cell activation (1). We have set up an experimental system to study if intramolecular conformational changes accompany this process. Fluorescence resonance energy transfer between FITC-labeled anti-CD4 (OKT4 epitope) and phycoerythrin (PE) conjugated anti-CD4 (Leu3a epitope) was detected by measuring the quenching of the donor's mean fluorescence in the presence of the PE-labeled acceptor by a FACscan apparatus, using the Autocomp software for setting of the compensation for the optical leakages to the opposite detector. Several other pairs of antibodies directed against different T cell antigens were used as controls. Up to 30 % quenching of the donor fluorescence was detected on both CD3-activated and resting cells, as compared to the 0-5 % shifts found when energy transfer was measured between TcR/CD3 and CD4, Leu8 or Leu5b. The results were compared to parallel measurements on an ACAS 570 system adapting the donor photobleaching method (2). The bleaching kinetics of individual cells proved to be double-exponential. No differences were apparent between capped and non-capped regions of the same cell, in terms of bleaching kinetics. The transfer efficiencies were calculated from either the initial bleaching rates or normalized donor intensities.

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(2) Jovin, T.M. and Arndt-Jovin, D., in Microspectrofluorimetry of single living cells, Academic Press, 1987 (Eds: E.Kohen, J.S.Ploem, J.G.Hirschberg).

Tu-Pos456

FT-IR STUDY OF LIPID PHASE TRANSITIONS AND WATER LOSS IN INSECT CUTICLES. Allen Gibbs and John H. Crowe. University of California, Davis, CA 95616

The surface of insects is covered with a layer of lipids, mainly long-chain hydrocarbons and wax esters, which constitutes the main passive barrier to water loss in these animals. Lipid phase transitions are believed responsible for the rapid rise in cuticular permeability as temperature is increased. We used Fourier Transform Infrared spectroscopy (FT-IR) to study lipid phase transitions in insect cuticles. Gel-to-liquid crystalline phase transition temperatures (T_ms) in intact moults were slightly higher (~2°C) than those of extracted lipids. Melting temperatures were higher in cuticles from insect species inhabiting warmer and drier environments, correlating with the need to minimize water loss under these conditions. We found significant intra-individual lipid variation. For example, in grasshoppers (*Melanoplus sanguinipes*), wing lipids melted at 70°C, whereas body lipids melted at 45°C. We also developed techniques for measurement of water loss rates and cuticle lipid properties in the same individual grasshoppers. These have enabled us to begin detailed studies of the relationship between cuticular permeability and lipid physical properties. Supported by NSF grant DCB 89-18822.

Tu-Pos457

ACTIVATION KINETICS USED TO DETERMINE NUMBER OF BINDING SITES ON THE NMDA CHANNEL

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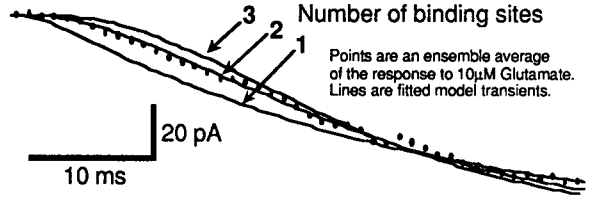
Excitatory synaptic currents in the CNS often exhibit a slow NMDA receptor mediated component which has a rise time of 8-10 ms and a decay time constant of 50-150 ms. Glutamate is probably the endogenous transmitter but glycine must also be present in order to activate this current. To understand the processes which produce this slow current, knowledge of the pharmacological and kinetic properties of the NMDA channel is required. Previous studies using whole cell recordings could not achieve solution changes in less than 10 ms, precluding detailed study of binding and activation kinetics.

We applied pulses of glutamate and glycine to outside-out patches of membrane from cultured hippocampal neurones by rapidly moving the interface of two flowing solutions across the tip of the patch pipette. Control experiments revealed that solution exchange at the surface of the membrane was complete in 1 ms.

Pulses of glutamate or glycine (0.2 to 20 μM) produced currents with slow, concentration dependent rise times. The time course of currents evoked by glutamate were accurately fitted using a multi-state kinetic model with two glutamate binding sites per receptor. Models with one or three binding sites per receptor could not accurately fit the data. A similar analysis of currents produced by glycine concentration steps is underway.



Supported by grants from NIH, ADAMHA and the McKnight Foundation (GLW).

Tu-Pos458

WITHDRAWN

Tu-Pos459

FLUORESCENCE SOLVENT ISOTOPE EFFECT IN INDOLE AND TRYPTOPHAN DERIVATIVES

Hong-Tao Yu, Lloyd P. McMahon, Mary D. Barkley
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Abstract

Excited-state proton exchange of indole and tryptophan derivatives in protic solvents has been proposed to be a fluorescence quenching pathway. We have studied the pH dependence of the solvent isotope effect of different indole derivatives, such as 3-carboxy-1,2,3,4-tetrahydrocarboline (**W(1)**), 3-Carboxy-3-amino-1,2,3,4-tetrahydrocarbazole (**W(2)**), 1,2,3,4-tetrahydrocarbazole (**W(1o)**), 1,2,3,4-tetrahydrocarboline (**W(1a)**), 3-carboxy-1,2,3,4-tetrahydrocarbazole (**W(1c)**) and 2-Me-indole. **W(1)** has a constant quantum yield in a pH(D) range of 4-8 and shows a double exponential decay, but does not show an isotope effect. **W(1a)** also does not show an isotope effect at pH(D) 7. Both **W(1)** and **W(1a)** have an isotope effect of about 1.6 at pH 10.5. **W(2)**, **W(1o)**, and **W(1c)** show an isotope effect of 1.5-2.0 at neutral pH. These results imply protonation on the indole ring during the fluorescence lifetime, since the positively charged amino substituent near the indole ring can prohibit this protonation and favor deprotonation. This is supported by the results: (1) many ammonium salts like hydroxylamine, glycine, and hydrazine are efficient fluorescence quenchers of indole derivatives, (2) the solvent isotope effect increases or decreases if indole is substituted by electron withdrawing or donating groups, respectively. 2-Me-indole shows no photo-initiated proton exchange in MeOH(D)/H(D)2O, but has an isotope effect of 1.5. Therefore, we propose that the pH independent solvent isotope effect of indole derivatives in the large pH 3-11 range is due to the attack by a solvent molecule at the N-lone pair of the excited, hydrogen-bonded indole. This destroys the conjugation and quenches the fluorescence, with no visible proton exchange. Further experiments to investigate this mechanism are in progress.
(Supported by NIH Grant GM42101)

Tu-Pos460

CONFORMATION AND DYNAMICS OF BOVINE BRAIN S-100A PROTEIN DETERMINED BY FLUORESCENCE SPECTROSCOPY.

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The effects of pH and Ca^{2+} on the environment of the single tryptophan residue 90 in bovine brain S-100a protein have been investigated using time-resolved fluorescence decay and steady state intensity quenching by acrylamide. A cavity-dumped dye laser synchronously pumped by a mode-locked Nd:YAG laser was used to excite the tryptophan. To minimize interference by tyrosyl fluorescence, the protein was excited and its emission was detected at 300 and 360 nm, respectively. At pH 7.2 the anisotropy decay function for apo-S-100a protein was best represented by a sum of two exponential terms yielding two rotational correlation times, $\phi_1 = 1.1$ ns and $\phi_2 = 16.2$ ns. The short component (ϕ_1) is attributed to the tryptophan side chain motion, rotating within a cone of semiangle 32°. The long component (ϕ_2) reflects the overall motion of the protein. The rotational time of an equivalent hydrated rigid sphere with a partial specific volume of 0.73 cm^3/g and a 20% hydration is 8.3 ns. The present result suggests that S-100a protein is not highly symmetric at pH 7.2. Ca^{2+} binding to the protein resulted in an increase in both correlation times and a decrease in the angular range of the side chain motion, suggesting that Ca^{2+} binding not only induced conformational changes but also slowed down the rotational rate of the protein and reduced the mobility of the tryptophan side chain. When the pH was changed to 8.4, the rotational correlation times became 1 and 18 ns with a rotational semiangle of 38°, suggesting an increase in rotational freedom for the fluorophore. In the Ca^{2+}-saturated state and at pH 8.4, only a single mode of rotational motion was observed. This result suggests that at the higher pH the mobility of the tryptophan side chain is either absent or too fast to be detected. Acrylamide quenching results showed that the single tryptophan residue was highly accessible and the Stern-Volmer plots were always linear, suggesting the absence of static quenching. (Supported by NIH AR25193 and M.R.C. Group, Canada).

Tu-Pos461

NONFLUORESCENT CONFORMERS OF PROTEINS AND TRYPTOPHAN AS REVEALED BY LIFETIME AND QUANTUM YIELD MEASUREMENTS.

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In 1971, DeLauder and Wahl showed that the single-tryptophan protein, human serum albumin (HSA) had multi-exponential fluorescence decay, suggesting that conformers of differing lifetimes coexist in solution. An extension of this idea is that some conformers of proteins are non-fluorescent; this hypothesis was tested by measuring protein lifetimes and quantum yields. The ratio of τ_m/Q_{rel} was obtained, where τ_m is the mean, intensity-weighted, lifetime, and Q_{rel} is the quantum yield relative to that of tryptophan. τ_m/Q_{rel} was 3.05 for tryptophan and ranged from 2.90 to over 20 for 25 different Trp-proteins. For melittin and HSA, τ_m/Q_{rel} were 10.1 and 8.8, indicating over 50% static quenching. Similarly, for some proteins and peptides containing Tyr but no Trp, the lifetime:yield ratio was much higher than that of free Tyr. These results are evidence for static self-quenching in proteins. Static self-quenching occurs in rotamers of Trp and related indoles; their natural lifetimes, as calculated from their ^1L_a bands (resolved according to Valeur and Weber) by the method of Birks and Dyson, are shorter than those calculated from lifetime and quantum yield data.

Tu-Pos462

COMPARISON OF THE ROTATIONAL ISOMERIC STATE MODEL FOR ALKYL CHAIN CONFORMATIONAL HETEROGENEITY WITH DISTANCE DISTRIBUTIONS DERIVED FROM FREQUENCY-DOMAIN FRET MEASUREMENTS ON A SERIES OF DIFFERENT FÖRSTER DISTANCE COMPOUNDS.

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Molecules of the form tryptamine-(alkyl-linker)-acceptor, in which the acceptor chromophore is varied to yield different Förster distance (R_o) values, were used to recover the shape of the end-to-end distance distribution. By globally analyzing the time-resolved energy transfer measurements for different members of the group together, each molecule can contribute to the refinement of the fitted donor-to-acceptor distance distribution function over the vicinity of its R_o value where it is most sensitive. Extending previous work which used steady-state measurements (Wiczk *et al.*, submitted to *Biopolymers*), the frequency-domain measurements provide a confirming perspective on the shape of the fitted distance distribution function.

In the rotational isomeric state model of polymer conformation, the probability of each bond rotational position depends on the rotational position of its neighbor bonds. Using a potential function determined for alkyl and amide bonds, a representative set of conformations was used to generate a distance distribution histogram for the specific linker used in the set. This histogram is nearly in agreement with the truncated, skewed Gaussian probability curve determined from a global (3-parameter) fit of the frequency-domain data. With a small adjustment of the choice of 'endpoint' positions within the donor and acceptor chromophores, the time resolved energy transfer measurement predicted by the histogram agrees with the measured frequency responses.

Tu-Pos463

STUDIES OF Tb^{3+} LUMINESCENCE AMONGST MUTANTS OF Ca^{2+} BINDING PROTEINS - ENGINEERING A HIGHLY LUMINESCENT Tb^{3+} BINDING PROTEIN.

C.W.V. Hogue[1,2], A.G. Szabo[1], J.P. MacManus[1], and D. Banville[3]. [1]Division of Biological Sciences, National Research Council, Ottawa, Ont. Canada, K1A OR6. [2]Graduate Student, University of Ottawa. [3]Biotechnology Research Institute, National Research Council, Montreal, Quebec, Canada, H4P 2R2.

The Ca^{2+} binding proteins parvalbumin, oncomodulin, and calmodulin all bind the isomorphous Ca^{2+} analogue, Tb^{3+}. The luminescence of Tb^{3+} can be enhanced by energy transfer from an aromatic amino acid such as Trp or Tyr which is within 10 A of the bound Tb^{3+}. Hence Tb^{3+} is used as a sensitive luminescent probe of the structure and interactions of these proteins. Using a series of site-directed mutants of oncomodulin, the effects of changing the molecular environment of the Tb^{3+} on its luminescence was studied and quantified. The amino acid ligand of the Tb^{3+}, the type of nearby aromatic amino acid and its position in the protein were examined. These results were compared to those obtained from other Ca^{2+} binding proteins. The oncomodulin mutants were shown to bind 2 Tb^{3+}, with the EF site filling first, and the CD site second. Studying the mutants showed Trp in a single loop at ligand position 7 best enhanced Tb^{3+} luminescence. Trp in any other position was not as effective at enhancing Tb^{3+} luminescence. When Phe or Tyr occupied position 7 of the binding loop they were responsible for the energy transfer enhancement of the Tb^{3+} luminescence. A cassette mutant oncomodulin with an altered binding site was investigated. A 10 residue stretch, comprising the CD loop, was substituted with a loop based on Tb^{3+} binding to model peptides. The order of fill reversed, such that the mutated CD site bound Tb^{3+} first. In addition, Tb^{3+} luminescence enhancement was greatly amplified. The detectability of this protein (10^{-11}) and a very long luminescent lifetime suggests it may have applications as a probe molecule. These studies show it is possible to engineer a highly luminescent Tb^{3+} binding protein from native Ca^{2+} binding proteins.

Tu-Pos464

BINDING OF 4,6-DIAMIDINO-2-PHENYLINDOLE WITH BOVINE SERUM ALBUMIN.

P. Cavatorta, R. Favilla, A. Mazzini, G. Sartor*, P. Neyroz, and A. G. Szabo#. Department of Physics, *Institute of Biological Chemistry, University of Parma, 43100 Parma, Italy, #Division of Biological Sciences, National Research Council, Ottawa, Ont. Canada, K1A OR6.

DAPI (4,6-diamidino-2-phenylindole) is a fluorescent dye which is known to bind to double stranded DNA, with a large enhancement of its fluorescence quantum yield. It has been used extensively as a fluorescent probe in structural studies of DNA. Preliminary results showed an increase of fluorescence intensity and changes in the static anisotropy of DAPI when the dye interacted with acidic proteins such as Bovine Serum Albumin (BSA), Pepsin, Human Serum Albumin (HSA), and Alcohol Dehydrogenase (YADH). In this work we report the binding parameters of DAPI bound to BSA (K_d = 100 uM; n = 3) measured by fluorescence titrations with the progressive dilution method. The pH behaviour and effect of salt indicate that the interaction is electrostatic. Moreover, the observe fluorescence decay time of DAPI (bound) (τ_1 = 2.83 ns; τ_2 = 0.97 ns), is similar to that reported for the binding to DNA. Similarly the correlation time (ϕ = 34 ns) obtained from anisotropy decay measurements confirmed that the dye binds to BSA. These results suggest that DAPI, employed in several staining procedures of DNA, could also bind to acidic proteins and be a useful fluorescence probe of these proteins.

Tu-Pos465

Aromatic Alcohols, Non-Aqueous Environments, Hydrogen Bonding and Excited-State Reaction Kinetics: Analysis Using Linear Combination of Spectra (LINCS).

E. Waxman, C.A. Hasselbacher, W.R. Laws, & J.B.A. Ross, Department of Biochemistry, Mount Sinai School of Medicine, New York, NY 10029

Compounds in their first excited electronic state may exhibit different properties compared to their ground state. Aromatic alcohols such as tyrosine and the estrogen 17β-dihydroequilenin become stronger acids in the excited state. Depending on the presence of a proton acceptor and the rates of deprotonation compared to the rates of excited-state decay, fluorescence may be observed in aqueous media from either the protonated or deprotonated alcohol, or both. To evaluate the potential behavior of aromatic alcohols, such as tyrosine, in a hydrophobic environment, such as the interior of a protein, we have examined their interactions with the strong proton acceptor triethylamine (TEA) in cyclohexane and toluene. The free alcohol and the hydrogen-bonded species exist in the ground state. While this population changes in the excited state, and formation of an ion pair may occur if the non-aqueous solvent can support one, completely dissociated ions are not found. We have shown that the absorbance and emission spectra at any [TEA] can be analyzed as a linear combination of spectra (LINCS). The basis set coefficients as a function of [TEA] were shown to fit a two-state, ground-state equilibrium model in which the populations of the two species change during the fluorescence lifetimes due to the differences between the depopulation rates of the two excited-state species. This analysis was followed by a parameter refinement process in which the rate constants for the system were fit for directly by the simultaneous analysis of all spectra. Those rate constants that could be verified by time-resolved fluorescence intensity decay measurements agree with the values determined from this analysis. We conclude that a stepwise approach, in which a model is selected for which the parameters obtained from different kinds of experiments are all consistent, followed by a global analysis procedure for parameter refinement, has general applicability for analysis of systems with complex ground-state and excited-state kinetics.

Supported by NIH Grants DK39548, GM12231, GM39750, and HL29019.

Tu-Pos466

NAPH-PE - LUCIFER YELLOW LOADED VESICLES: ENERGY-TRANSFER STUDIES TO FOLLOW THE PHOSPHOLIPID TRANSPORT AT THE INNER BILAYER SIDE.

M. Truzzi, N. Calonghi and P. Neyroz

Istituto di Chimica Biologica, Universita' di Parma, Italy.

The excitation and emission properties of different fluorescent dyes have been used to follow the incorporation of 2-naphthol labeled phosphatidylethanolamine (NAPH-PE) into liposomes. Unilamellar phospholipid vesicles were prepared by the detergent-dialysis method and loaded with the polar fluorescent probe lucifer yellow. The dye has an excitation maximum at 420 nm (λ_{min} 332 nm) and an emission maximum at 520 nm, and is suitable to accept resonance energy-transfer from the emission at 410 nm of naphtholate-PE as a donor. The NAPH-PE deprotonated species fluorescence was induced by performing all the experiments in the presence of 0.2 M sodium acetate and exciting the sample at 334 nm. Under this conditions, preliminary results have been obtained that suggest that complete phospholipid translocation take place in 3-4 hours at 28° C. To confirm this data, additional vesicles preparation have been carried out in which DPH-PC was inserted into the bilayer. The diphenylhexatrienyl chromophore is excited at 362 nm and emits at 433 nm, thus providing an intermediate step in the energy-transfer process between lucifer yellow and protonated NAPH-PE. This combination of fluorophores should provide an energy-transfer cascade ideal for overdetermination of lipids translocation kinetics.

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Tu-Pos467

FLUORESCENCE STUDIES OF OSPA, THE OUTER SURFACE PROTEIN FROM THE LYME DISEASE SPIROCHAETE, BORRELIA BURGDORFERI. J. Kieleczawa, L.L. France, J.J. Dunn, G. Hind and J.C. Sutherland. Biology Dept., Brookhaven National Laboratory, Upton, NY 11973.

Using steady-state and time-resolved fluorescence techniques, we studied the environment of the single tryptophan (trp-216) residue in outer surface protein A (ospA) from the spirochaete, Borrelia burgdorferi, the etiological factor in Lyme disease. At pH 7.1, trp-216 appears to be buried within a hydrophobic domain of the protein, as judged by the trp emission maximum (330 nm). Furthermore, the fluorescence is easily quenched by a neutral quencher, acrylamide (k_q = $1.93x10^9M^{-1}s^{-1}$), but trp-216 is almost inaccessible to the ionic quenchers I^- and Cs^+ (k_q = $0.37x10^9M^{-1}s^{-1}$ and k_q = $0.19x10^9M^{-1}s^{-1}$, respectively). Upon denaturation by guanidinium-HCl (6 M), the efficiency of all three quenchers increases, as expected (k_q = $5.58x10^9M^{-1}s^{-1}$, k_q = $1.67x10^9M^{-1}s^{-1}$ and k_q = $0.51x10^9M^{-1}s^{-1}$, respectively). OspA can be fully denatured by either high temperature (>60°C) or by Gdn-HCl (≥1.0 M). In both cases, the fluorescence intensity at 330 nm decreases and the λ_{max} is shifted to 352 nm. Upon removal of the denaturing factors, the protein returns to the native state, as judged by the observation that the fluorescence intensity and the λ_{max} return to their initial values. At pH's between 5 and 11, λ_{max} remains stable (330 nm), and only a small decrease (< 15%) in fluorescence intensity (at 330 nm) is observed at both extremes (pH 5 and pH 11); the decrease in fluorescence intensity is fully reversible, after adjustment to neutral pH. The fluorescence lifetime of trp in native ospA (1.12 ns) shows no significant change over a range of NaCl concentrations (zero to 1 M). From these experiments we conclude that: (1) ospA is stable over a wide range of pH conditions and NaCl concentrations; (2) the transitions, native ⇌ denatured, are apparently entirely reversible.

Supported by the Offices of Basic Energy Research and Health and Environmental Research, USDOE, and a NIH grant to JCS (GM34662).

Tu-Pos468

ELECTRONIC ENERGY LEVELS OF ETHENO-ADENOSINE

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Ethenoadenosine (εAdo) is one of the fluorescent derivatives of adenosine, used as a biophysical probe of nucleic acids and ATP.

Several models, based on protonation and deprotonation, were proposed to explain the complex photophysics of εAdo. We suggest a scheme, in which an interplay of nπ* and ππ* states predominates. The model explains known facts, the interpretation of which was subject to debate and, in addition, our measurements of : 1) Fluorescence of εAdo adducts with ß-Cyclodextrin and 2) Room temperature phosphorescence.

Tu-Pos469

A BIFURCATING ULTRAMETRIC DISTRIBUTED SYSTEM (BUDS) TO MODEL THE THERMAL BEHAVIOR OF SINGLE TRYPTOPHAN PROTEIN FLUORESCENCE.

N. Silva and E. Gratton. *Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, 1110 W. Green Street, Urbana, IL 61801.*

A dynamical model describing protein motion can be useful in predicting protein function. Protein fluorescence is a sensitive experimental technique which gives information on the nanosecond dynamics of intrinsic aromatic side chains, particularly tryptophan. Due to the complex nature of proteins and our present analytical abilities, single tryptophan residue proteins are studied. In order to obtain information about the energetics of the protein system, such as activation barriers, fluorescence time decay experiments are performed as a function of temperature. The fluorescence decay of single tryptophan proteins displays a complex behavior as a function of temperature. The time-resolved fluorescence decay of such proteins had been described as a distribution of decay rates whose width increases with decreasing temperature. This form and behavior of the fluorescence time decay is attributed to the existence of conformational substates in proteins. The BUDS model explores temperature dependent fluorescence decay of single tryptophan proteins. BUDS introduces conformational substates through an ultrametric distribution of hierarchical activation barriers separating these substates. The key feature of BUDS is that it predicts unconventional non-exponential behavior of the fluorescence time decay at low temperatures. We have investigated the similarities of the BUDS model with protein fluorescence behavior as a function of temperature and its physical interpretation. Finally, we have applied BUDS to the temperature- and denaturant-dependent fluorescence lifetime experiments of human superoxide dismutase. This work is supported by NIH grant PHS-P41-RR03155.

Tu-Pos470

PARALLEL MULTI-CHANNEL TIME-RESOLVED AND STEADY-STATE FLUORESCENCE SPECTROSCOPY: APPLICATION TO PROTEIN/DNA INTERACTIONS AND PROTEIN FOLDING PROBLEMS. Joseph M. Beechem, Vanderbilt University, Dept. of Molecular Physiology and Biophysics, Nashville TN 37232.

Recent work [Beechem, *Biophys.J.* 57:430a, 1990] described the construction of a 4 channel parallel time-resolved fluorescence spectrometer based on the utilization of a 10 channel multi-anode microchannel plate detector and multiple TAC's. This instrument has been further upgraded so that 8 full parallel detection channels are now operational. In addition, an ultra-small volume sample chamber (with miniaturized lenses and fiber optics) has been developed so that measurements can be routinely performed on 12 μℓ samples. All of this instrumentation is interfaced to a stopped-flow apparatus and optical multichannel analyzer. The software controlling this instrument has now been developed so that 8 time-resolved fluorescence decay curves can be collected every 10 milliseconds, and 512 pixel element steady-state emission spectra (covering either 125 or 256 nm regions) can be obtained every 1.0 millisecond. Summing data over 50 repeat stopped-flow runs, allows full decay curves (one million total counts) to be collected every 10 millisecond time slice. Utilizing a specially modified stopped-flow apparatus from Molecular Kinetics™ (Pulman, WA), these 50 repeats can be performed using as little as 250 μℓ of total material. Application of this technology towards the collection of very high dimensional data consisting of: fluorescence *vs* time (both picosecond and millisecond) *vs* emission wavelength *vs* quencher *vs* polarization *vs* ligand will be described. The global analysis routines are being enhanced to allow the simultaneous incorporation of two time-scales directly in a single analysis. Stopped flow studies of protein:DNA interactions in transcription factor TFIID and bacterial repair enzyme Endonuclease V will be presented. Protein folding studies on *Staphylococcal Nuclease* will also be described. JMB is a L. P. Markey scholar in biomedical science.

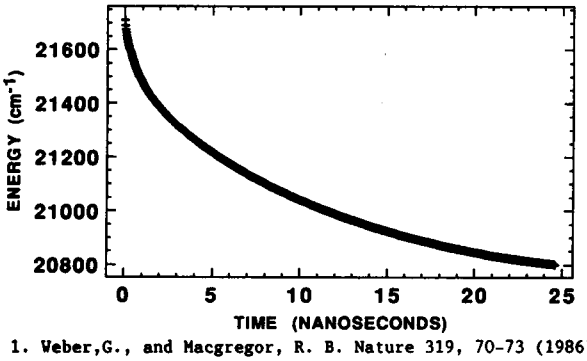
Tu-Pos471

DIELECTRIC RELAXATION IN A PROTEIN MATRIX. Daniel W. Pierce and Steven G. Boxer, Department of Chemistry, Stanford University, Stanford, CA 94305.

We report measurements of the time-dependence of the Stokes shift in the complex formed between apo-myoglobin and 2'-(N,N-dimethyl)amino-6-napthyl-4-trans-cyclohexanoic acid (DANCA) [1] on the picosecond timescale. The relaxation of the mean emission energy is shown below. If this decay is fit to a Debye form, at least three longitudinal relaxation times τ_L are required. It is more likely that the decay is nonexponential with a frequency spectrum spanning the experimentally observable (40 GHz to 40 MHz) range. This would be consistent with a model in which protein modes over at least this range contribute significant amplitude to the relaxation.

The rates of condensed phase electron-transfer reactions can depend on the same dynamics as the Stokes shift. The large (18 Debye) difference dipole of this dye provides a perturbation with easily measured effects, and the lack of other complicating excited-state processes yields a decay curve that is directly relevant to the modeling of biological electron transfer.

DECAY OF EMISSION ENERGY



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Tu-Pos472

LOW VISCOSITY OF THE FLUID-PHASE CYTOPLASM MEASURED BY MULTI-HARMONIC FREQUENCY-DOMAIN POLARIZATION MICROFLUORIMETRY. *Kiyohide Fushimi and A. S. Verkman, CVRI, UCSF, San Francisco. CA.*

Information about the rheological characteristics of the fluid-phase cytoplasm is provided by analysis of the rotational motion of polar molecules in the cell. To determine fluid-phase cytoplasmic viscosity in intact Swiss 3T3 fibroblasts, a time-resolved polarization microscope system was constructed. Fluorophores in the cytoplasm were excited by a pulsed Ar-laser light and differential phase angles and modulation amplitude ratios of emitted light at modulation frequencies of 5-300 MHz were obtained in parallel by multi-harmonic cross-correlation detection. Data were fitted to a two-component anisotropic rotational model. We found that the shorter rotational correlation time (τ_{1c}) of the probes, 2,7-bis-(2-carboxyethyl)-5-(and-6-)carboxy-fluorescein (BCECF), 6-carboxyfluorescein (6-CF), and 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS, pyranine) provided a direct measure of cytoplasmic viscosity which was independent of probe binding. In quiescent fibroblasts, τ_{1c} values (294 $\pm$ 7 ps, BCECF; 180 $\pm$ 20, 6-CF; 181 $\pm$ 12, HPTS) were 20-40% longer than those in free water (241 $\pm$ 5 ps, BCECF; 131 $\pm$ 7, 6-CF; 127 $\pm$ 7, HPTS), indicating that the cytoplasm was only 1.2-1.4 times as viscous as free water. The activation energy of cytoplasmic viscosity was 4 kcal/mol, similar to that of free water. Cytoplasmic viscosity was altered by <10 % upon addition of vasopressin to activate phospholipase C, sucrose to decrease cell volume, and cytochalasin B to disrupt cell cytoskeleton. Nucleoplasmic and peripheral cytoplasmic viscosities were not different. These results establish methodology to measure picosecond time-resolved fluorescence in subcellular domains of living cells. Our data indicate that the physical properties of fluid-phase cytoplasm are similar to those of free water in simple protein solution.

Tu-Pos473

EFFECT OF pH ON RATE CONSTANTS AND ION SELECTIVITY OF FLUORESCENT DIVALENT CATION INDICATORS. Frank A. Lattanzio, Jr. and Dieter K. Bartschat. Eastern Virginia Medical School, Norfolk, VA 23501.

Fluorescent calcium indicators such as fura-2, indo-1 and fluo-3 have a reduction in affinity at acidic pH as determined by an increase in the apparent calcium dissociation constants (Kd) (F. Lattanzio, BBRC 171:102, 1990). To determine whether these changes were caused by alterations in either on- and/or off-rates of indicators, both the Kd and off-rates of fura-2, indo-1 and fluo-3 and the magnesium indicators mag-indo-1 and mag-fura-2 were measured, permitting calculation of the on-rate. Both calcium and magnesium measurements were made to determine if ion selectivity changed as pH declined. For stop-flow experiments, the indicators were placed on 0.1M KCl, 40 mM HEPES with pH at 7.40 or 7.00 or 40mM MES at pH 6.50, 6.00 and 5.50 with calcium or magnesium and mixed with a KCl-EDTA buffer. This buffer system was also used to measure the Kd. The off-rates for calcium of mag-into-1 and mag-fura-2 and for magnesium of fura-2, indo-1 and fluo-3 were too rapid to be resolved (t1/2<0.1 msec). The off-rates for magnesium of mag-fura-2 and mag-indo-1 were measured at 10 C to permit accurate resolution of these values. All other measurements were at 22 C. We conclude that the calcium on-rates for fura-2, indo-1 and fluo-3 increases more rapidly than the slowing of the off-rate, causing the Kd to increase and the affinity to decline. The calcium:magnesium selectivity ratio of indo-1 and fura-2 is altered in acidic environments. These factors will affect measurements of calcium transients and intracellular calcium and magnesium activity during pathological conditions such as ischemia.

Tu-Pos474

STUDIES OF THE EFFECT OF HYDROGEN BONDING ON THE ELECTRONIC AND EMISSION SPECTRA OF ALL-TRANS-RETINAL AT ROOM-TEMPERATURE. S. Alex, H. Le-Thanh and D. Vocelle, Département de chimie, Université du Québec à Montréal, C.P. 8888, Succ."A", Montréal, Québec, H3C 3P8, Canada.

Studies at room-temperature of electronic and emission spectra of all-trans-retinal exposed to increasing amount of trifluoroacetic acid (TFA hereafter) have been achieved in various solvents in order to characterize the nature of the H-bonded complexes formed in the ground and excited states. Depending on the electrostatic properties of the solvents and the state concerned, the position of the hydrogen atom is differently located along the axis of the C=O....HOOC bridge. In apolar and aprotic solvents (ie. hexane) as well as in protic and polar solvents (ie. methanol) all-trans-retinal forms moderate and well-defined H-bonds with TFA in the ground state, except in large excess of H-bonding agent where a ionic system transfer from the carboxylate function towards the carbonyl group is quickly achieved, whatever the initial concentration of both solutes and only the ionic system is observed. This is deduced from the large Stokes shift (ca. 10000 cm^{-1}) monitored between the excitation and emission spectra of these compounds and also from the fact that the fluorescence of retinal is reputed to be absent at room-temperature unless the energy level ordering is sufficiently affected by external perturbations like, for example, a proton transfer (J. Papnikolas, G.C. Walker, V.A. Shamanian, R.L. Christensen and J. Clayton Baum, J. Am. Chem. Soc. 112, 1920-1921, 1990 and R.R. Birge, Ann. Rev. Biophys. Bioeng. 10, 315-354, 1981). This situation is particular to these two types of solvents, since in solvent moderately polar and having or not a slight H-bonding capacity (ie. chloroform and dichloromethane respectively) this behavior is not observed. In these latter cases, all-trans-retinal is involved in H-bonds whose strength is very dependent on the TFA concentration, and this leads to the formation of differently H-linked all-trans-retinal molecules and aggregates in the ground state. Then, no proton transfer is observed in the excited state and the molecules offer a poor resistance towards light exposure, generating highly coloured charged by-products, this being consistent with the fact that a facile H translation is the key to stability. From these experiments, we believe that H atom transfers are easily carried out only when the molecules are weakly solvated (ie. in hexane) or when the ionic form is easily stabilized (ie. in methanol), otherwise they are severely reduced.

Tu-Pos475

Metal ion effects on indole luminescence in an indole-EDTA chelator.

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The indole fluorescence of an indole-EDTA chelator is markedly quenched by paramagnetic metal ions including Cu(II), Mn(II), Co(II), and several lanthanides. Notably, Tb(III) binds with a stoichiometry of 1, quenches 55% of the indole fluorescence, and exhibits typical Tb luminescence through resonance energy transfer when the indole is pumped. Oxygen and acrylamide quench the fluorescence of the indole moiety with rates typical of their effects on other indoles, but the sensitized luminescence of the chelated Tb is quenched several hundred times faster. Iodide increases the luminescence of the chelated Tb. These results and measurements of fluorescence lifetimes demonstrate clearly that paramagnetic metal ions quench effectively by mechanisms operating through space and suggest that triplet and singlet states probably contribute to indole-Tb energy transfer. Supported by GM34847.

Tu-Pos476

A NOVEL REAGENT FOR FUNCTIONALLY-DIRECTED SITE-SPECIFIC FLUORESCENT LABELING OF PROTEINS.

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Fluorescence analysis of protein structure and dynamics is often limited by the lack of selectivity of the site of labeling with fluorescent probes. To overcome this, we have synthesized a cleavable crosslinking reagent to specifically transfer a fluorophore from one protein onto the binding domain of a selected interacting protein. The reagent contains a terminal N-sulfosuccinimidyl ester to label the amino groups of the "donor" protein, a terminal photoactivatable azido-coumarin species to react with a proximal side chain of the interacting "acceptor" protein, and a central disulfide linker to release the labeled acceptor after cleavage. Photoactivation at 350 nm results in the rapid generation of both the reactive nitrene and coumarin fluorescence (λ_{ex}^{max} 350 nm, λ_{em}^{max} 450 nm, Q 0.6). To test the proposed labeling scheme, soybean trypsin inhibitor (STI) was reacted in the dark with a 3-fold excess of reagent and quickly isolated by gel filtration. The derivatized STI, which retained full activity, was then photolysed in the presence of a stoichiometric amount of trypsin. A fluorescent species (3 mol% of total STI) with the MW of a 1:1 complex of STI and trypsin was observed on SDS gels. The uncomplexed trypsin fraction was not labeled, whereas the trypsin recovered after reductive cleavage of the complex was labeled (~1 mol/mol). No transfer of label was observed when the reagent was not photoactivated or was cleaved before photolysis. Also, an excess of underivatized STI inhibited trypsin labeling. Finally, no transfer of label onto carbonic anhydrase, a non-interacting protein, was detected. These controls indicate the specificity of the labeling procedure. Given the low stoichiometry of STI derivatization (~0.3 mol/mol) and the absence of label on the STI recovered from the reduced complex, we conclude that a labeled STI transferred one coumarin moiety to trypsin with an overall efficiency of ~10%. The short (<15Å) crosslinking reach of this reagent limits the transfer to the vicinity of the contact region between the two proteins. Thus, this novel reagent permits the specific fluorescent labeling of a protein in its active form near a selected binding site of physiological importance.

Tu-Pos477

FLUORESCENCE DECAY KINETICS AND ENERGY TRANSFER IN OLIGONUCLEOTIDE-DYE CONJUGATES.

C. R. Guest, R.A. Hochstrasser, S.M. Chen, F. Heffron and D.P. Millar, Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 N. Torrey Pines Rd., La Jolla, CA 92037. The fluorescence decay kinetics of covalent oligonucleotide-dye conjugates has been examined by time-resolved fluorescence spectroscopy. Our eventual aim is to use fluorescence energy transfer to obtain structural information on DNA duplexes and Holliday junctions in solution. However, electronic interactions between the dyes and DNA must be accounted for before FET can provide quantitative distance information. Oligonucleotides were synthesized with dyes attached at the 5′ terminus via an aminoethyl linker. The fluorescence decay kinetics of oligonucleotide strands labelled with either fluorescein or tetramethyl rhodamine were examined. A complex multi-exponential decay was observed for both dyes and was found to depend on the length and base-sequence of the oligonucleotide strand. The decay kinetics changed when the labelled strand was annealled with an unlabelled complementary oligonucleotide, again in a sequence-dependent fashion. These results demonstrate that the dyes interact with the oligonucleotide and that the interactions are different in single-strand and duplex oligonucleotides. These interactions were also studied by time-resolved anisotropy decay, in which the rotational mobility of the dyes was observed. Duplexes were then formed from a fluorescein-labelled strand and its complement labelled with tetramethyl rhodamine. Energy transfer from the fluorescein donor to the tetramethyl rhodamine acceptor was measured by time-resolved donor quenching as a function of the strand length. The geometry of a 4-way branched Holliday junction containing 32 b.p. was determined by estimating the separation of pairs of 8 b.p. branches labelled with the donor and acceptor dyes, based on measurements of the energy transfer rate. The results were consistent with a planar, two-fold symmetric structure.

Tu-Pos478

DUAL EXCITATION, pH SENSITIVE CONJUGATES OF DEXTRAN AND TRANSFERRIN FOR pH MEASUREMENT DURING ENDOCYTOSIS UTILIZING 514 NM TO 488 NM EXCITATION RATIOS.

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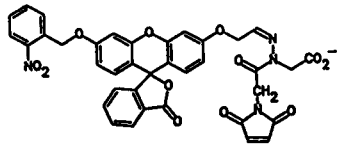
Fluorescent conjugates of dextrans and transferrin were prepared and evaluated as pH indicators for endocytic processes. The 70,000 Da dextran conjugates are membrane impermeant and eventually localize in lysosomes, while the iron binding protein transferrin is recycled to the plasma membrane. The fluorescent, pH sensitive dyes used were rhodol derivatives that have low pK_a values and greater photostability than fluorescein derivatives. The dyes and their conjugates are also suitable for dual excitation at 514 and 488 nm, wavelengths that match the principal emission lines of the argon ion laser, commonly used in confocal microscopy and flow cytometry. Excitation spectra of these derivatives show a clear change in bandshape as a function of pH, but lack a true isosbestic point. The ratio of excitation at 514 nm relative to 488 nm is pH dependent. The pK_a values of the pH indicators ranged from approximately 4.5 to 6.0, making them suitable for pH determination in acidic media between approximately pH 3.5 and 7.0. Quantum yields of the free dyes ranged from approximately 0.8 to 0.9 in aqueous solution, with emission maxima between 535 nm and 540 nm. The pH sensing properties of the dyes were retained subsequent to conjugation with the protein or polysaccharide. These fluorescent probes are suitable for dual excitation pH measurement under acidic conditions using argon ion laser based methods. This work was supported in part by NIH grant GM37347.

Tu-Pos479

A PHOTOACTIVATABLE FLUOROPHORE FOR LABELLING THIOLS: MODEL STUDIES WITH MYOSIN LIGHT CHAIN-1. John E.T. Corrie[1], Yale E. Goldman[2], and David R. Trentham[1], [1]National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K. and [2]Department of Physiology, University of Pennsylvania, Philadelphia PA 19104.

A derivative of fluorescein is described which is non-fluorescent until photochemically cleaved. The fluorescence is blocked by alkylation of both phenolic groups of the fluorescein molecule, of which one bears a photosensitive o-nitrobenzyl substituent. The other phenolic group bears a substituent which incorporates a carboxylic acid to promote solubility in aqueous media and a maleimide as a site for labelling macromolecules via thiol addition. Brief incubation (0.5 h at pH 7.4) of the compound (0.8mM) with myosin light chain-1 from rabbit skeletal muscle gives specific attachment, as demonstrated by blockage of the reaction via pretreatment of the protein with N-ethylmaleimide. Non-covalently bound reagent can be removed by a combination of gel filtration and anion-exchange f.p.l.c. Irradiation of the labelled protein with a pulse of near-ultraviolet light cleaves the o-nitrobenzyl group which results in the labelled protein becoming fluorescent. This and other proteins labelled with the new reagent are suitable for resolving spatial problems in vivo by virtue of the potential for localised release of fluorescence by photolysis (cf. Mitchison (1989) J. Cell Biol., 109, 637-652).

Supported by the Medical Research Council (UK) and N.I.H. grant HL15835 to the Pennsylvania Muscle Institute.



Tu-Pos480

FLUORESCENCE STUDY OF A MUTANT CYTOCHROME B5 WITH A SINGLE TRYPTOPHAN IN THE MEMBRANE-BINDING DOMAIN. Alexey S. Ladokhin, L. Wang*, A. W. Steggles* and Peter W. Holloway. Dept. of Biochemistry, Univ. of Virginia Sch. Med., Charlottesville, VA. *Dept. of Biochemistry, Northeastern Ohio Univ. Coll. Med., Rootstown, OH.

Cytochrome b_5, isolated by detergent extraction from rabbit liver, has been extensively studied by fluorescence techniques, however its fluorescence properties are complicated by the presence of three Trp in the membrane-binding domain. This protein has now been expressed in E. coli and a mutant form has been isolated (108^-, 112^-) which contains only one Trp (109) in the membrane-binding domain. This mutant behaves similarly to the native protein: it binds to lipid vesicles with an enhancement of fluorescence and is quenched to the same extent as the native protein by a series of brominated phospholipids. These data suggest that the membrane binding domain with two Leu substituted for the two Trp has a very similar topography to that of the native protein in the membrane. The mutant protein does differ from the native protein in several of its spectroscopic properties. The mutant protein has a quantum yield (per molecule of protein) of 80% of that of the native form and has a blue shifted emission spectrum compared to the native form. Fluorescence anisotropy measurements of the proteins bound to detergents show that the native protein has a greater degree of depolarization, which is compatible with nonradiative energy transfer involving the three Trp of the membrane-binding domain. Both proteins show a red shift of the emission spectrum under conditions of red-edge excitation. This suggests that the fluorescence from both proteins occurs from a tryptophan excited state before the fluorophore has reached complete equilibrium with its dipolar environment and that there exists a distribution of microstates with rates of dipolar relaxation which are comparable to the fluorescence lifetime. Supported by GM 23858.

Tu-Pos481

EQUILIBRIUM DYNAMICS OF MELITTIN STUDIED BY INTRINSIC FLUORESCENCE.
Alexey S. Ladokhin*, A. Yu. Chikishev, V. F. Kamalov, N. V. Lebedeva, B.N. Toleutaev. (Intro. by W. R. Pearson+.) *A.V.Palladin Inst. of Biochemistry, Ac. Sci. UkSSR, Kiev, Ukraine. Moscow State Univ., USSR. +Dept. of Biochemistry, Univ. of Virginia Sch. Med.,Charlottesville, VA.

The structural dynamics of a single tryptophan protein, melittin, have been studied by means of fluorescence spectroscopy over a wide temperature range. Blue spectral shifts in intrinsic fluorescence which are observed at low temperatures (<-10° C) are attributed to the conformational changes involving the dissociation of tetrameric molecule. In contrast red shifts at high temperatures (>+25° C) are found not to be associated with cooperative transitions and are caused by an increase of dipolar mobility in the native structure. At intermediate temperatures the dynamics of Trp-19 environment are slow (on a nanosecond scale) hence the emission is from structurally nonequilibrium excited states. To fit the data the Bakhshiev-Mazurenko model for continuous monoexponential relaxation was used with a modification to account for the existence of a distribution in interaction energies of the chromophore with surrounding dipoles. The parameters of the structural dynamics of the Trp-19 environment in melittin calculated from experiments with time resolution and steady-state red-edge excitation have similar values. Iodide quenching and quantum yield measurements show that the activation energy of the equilibrium structural fluctuations in tetrameric melittin, which allow both external quenchers and quenching groups of protein itself to interact with the tryptophanyl, is 10-15 kJ/mole. The activation energy of reorientation of internal dipoles in melittin is higher (30 kJ/mole.)

Tu-Pos482

THE EFFECT OF TIGHT ACTIVE-SITE BINDING MOLECULES ON THE ORGANOMERCURIAL PROMOTED RELEASE OF ZINC IN HLADH: CONFORMATIONAL STUDIES WITH FLUORESCENCE.
Carole J. Spangler and Ludwig Brand, Biology Dept., The Johns Hopkins University, Baltimore, MD 21218.

Horse liver alcohol dehydrogenase (HLADH) is a dimer of identical subunits, each monomer containing one catalytic and one structural zinc atom. Previously, we have shown that the specific structural role of the second zinc atom is to stabilize the proper dimer structure. Without this zinc atom, the molecule can still exist in a dimeric form, but it is distinctly different from the native dimer. To further probe the effect of this zinc atom on monomer-monomer interactions, tight active-site binders (NAD+ and pyrazole) were bound to the native protein, and zinc release was studied as initiated with p-mercuriphenylsulfonate (PMPS), a sulfhydryl reagent. These active-site binders stabilized the "native" HLADH dimeric structure, as the conformational change initiated by the PMPS reagent took place over approximately 60 minutes instead of 10 minutes as observed under native conditions. The reaction was monitored by a decrease in tryptophan steady-state fluorescence intensity, a decrease in steady-state and time-resolved fluorescence anisotropy, changes in relative amplitudes associated with fluorescence lifetimes characteristic of each conformational species, and changes in decay associated spectra. High pressure liquid chromatography was used to verify a model for the quarternary structure changes. (Supported by NIH Grant #GM11632, CJS supported by NSF Grant #DIR 8721059).

Tu-Pos483

CHANGES IN DISTANCE DISTRIBUTION OF A STAPHYLOCOCCAL NUCLEASE MUTANT DURING GUANIDINIUM UNFOLDING AS DETERMINED BY FLUORESCENCE ENERGY TRANSFER

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The protein from a mutant clone of Staphylococcal nuclease (obtained from David Shortle) with a cysteine substituting for a lysine at position 78 was prepared and labeled with the cysteine specific fluorescent probe 5-(2-(iodoacetyl)amino)ethyl)-amino)naphthalene-1-sulfonic acid (1,5-IAEDANS). Time resolved non-radiative energy transfer studies were done using the single trp-140 as the energy donor and the 1,5-IAEDANS as the acceptor. Changes in distance and distance distributions were observed as a function of increasing guanidinium concentration (0-2 M) and in the presence or absence of the inhibitor pdTp and Ca^{2+}. In the native state, both the ternary complex and non-complexed protein are best fit with one population having an average donor acceptor distance of about 23 Å and a full width at half maximum (FWHM) of about 18 Å. During GuHCl unfolding, the average distance remains relatively constant up to the unfolded state for both the ternary complex and the free protein. In the unfolded state, the FWHM of the free protein is about half that of the native form. The FWHM increases to a larger value as the protein is further denatured in 2 M Gu-HCl. In all cases, one population is sufficient to fit the decay data of the free protein. In contrast, the FWHM of the ternary complex remains constant up to unfolding. The FWHM of the denatured state is greater than that of the native. At 2 M Gu-HCl, two populations are required to fit the data, one population with a compact structure and the other with an expanded structure. The influence of pdTp(Ca^{2+}) can be seen even in the non-native configuration perhaps continuing to stabilize an as yet undefined structure or structures. (Supported by NIH grant #GM 11632).

Tu-Pos484

FLUORESCENCE INTENSITY AND EMISSION ANISOTROPY DECAY OF DIPHENYLHEXATRIENE IN SINGLE BILAYER PHOSPHOLIPID MEMBRANES.

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It is well known that the decay of the total intensity and the emission anisotropy of 1,6-diphenyl-1,3,5-hexatriene in single bilayer phospholipid vesicles is complex. The former has been interpreted in terms of complex photophysics, photochemistry or distribution of the probe in different domains. The latter has been interpreted in terms of hindered rotation or other anisotropic features of the bilayer membrane.

We now describe a single theory which accounts for both the fluorescence intensity and anisotropy decays. It is based on the known fact that when a chromophore is in one medium and separated by a small thickness from a medium of a different refractive index, the absorption and emission probability should depend on the orientation of the chromophore (Lukosz, W., Phys. Rev. B, 22, 3030 (1980). For instance, fluorescence lifetimes of probes in liposomes should be different for different probe orientations, which should lead to intensity decay other than that expected in a continuous media such as an oil. The model also takes into account the angular diffusion of excited probe molecules. Comparisons between the model predictions and experimental data will be described. (supported by NIH grant No. GM11632)

Tu-Pos485

ORIENTATION OF HUMAN CD59 UPON INSERTION INTO THE PHOSPHOLIPID BILAYER. A FLUORESCENT RESONANCE ENERGY TRANSFER STUDY.

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CD59 is a potent inhibitor of the terminal complement proteins that is tethered to the plasma membrane via a glycan-phosphatidylinositol anchor. Amino acid sequence analysis of CD59 revealed the presence of a single tryptophan residue located at position 40. We have investigated the spectral properties of Trp 40 in the native and denatured states of CD59 as well as the spatial relationship of Trp 40 to the membrane surface when the protein is tethered to the phospholipid bilayer. In the presence of 6 M guanidine-HCl, Trp 40 emission maximum exhibited a red shift from 336 nm to 354 nm. Polarization measurements upon titration of native CD59 with guanidine-HCl revealed complete unfolding at ≥ 3 M [guanidine-HCl]. Steady state collisional quenching experiments using acrylamide and I⁻ yielded Stern-Volmer quenching constants of 1.65 and 0.9 M^{-1}, respectively, suggesting that Trp 40 is buried within the molecule. The distance of CD59 Trp 40 to the membrane surface was estimated by fluorescence resonance energy transfer (RET) to dansyl-DPPE. Trp 40 fluorescence did not change upon incorporation of CD59 into vesicles containing unlabeled lipid. In the presence of labeled phospholipid (0-20 mole% dansyl-DPPE), RET from Trp 40 to dansyl was detected by a decrease in tryptophan fluorescence with increasing mole-fraction of dansyl-DPPE incorporated into the vesicle membrane. At 20 mole-percent dansyl, RET efficiency of 59 ± 3% was observed. The distance of closest approach between Trp 40 and the dansyl moiety located at the membrane surface was estimated to be 25-26 Å, calculated using the equation of Dewey and Hammes (1980) *Biophys J. 32,* 1023-1035. Assuming a partial specific volume of 1.2 g/cm³ and a spherical geometry of 18 Å radius for the protein, these data suggest that CD59 is closely apposed to the membrane surface. *This work was supported by HL36061 and HL36946 from NHLBI and an Established Investigatorship Award from AHA.*

Tu-Pos486

FLUORESCENCE OF PYRENE-LABELED PHOSPHATIDYL-CHOLINE IN LIPID BILAYER MEMBRANE. A THREE STATE MODEL.

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In this paper, a *three state model* has been developed to describe the fluorescence of pyrene-labeled phospholipids in lipid membranes. The model has explicit solution in frequency domain and it is applicable in a wide range of probe concentrations. The *three state model* is a generalization of the Birks model (Birks et al., (1963) Proc. R. Soc. London A. Math. Phys. Sci. 275:575-588) which proved to be a failure in the case of membrane systems. In contrast to the Birks model, the *three state model* makes distinction between two types of the excited monomers: excited monomers which *have* and which do *not have* ground state probe molecules in their nearest neighbor. The presence of ground state probe molecules in the nearest neighbor of an excited monomer may result in *excimer* formation at proper orientations of the respective pyrene rings.

The kinetics of the fluorescence process is represented in the model by three, coupled, first order, linear differential equations with coefficients depending on the probe concentration. The analytical forms of the concentration dependencies of the coefficients are determined by means of an Ising type model of the two-component membranes. This combination of the reaction kinetical and statistical mechanical models describes properly the pyrene fluorescence in the cases of both ideal and non-ideal mixtures of pyrene-labeled probes and matrix lipid molecules.

By using the explicit solutions of the *three state model*, the *global analysis* of the phase-modulation fluorescence data provides information on the fluorescence rate parameters and on the static and dynamic parameters of the lateral distribution of the pyrene-labeled lipid molecules in the membrane. The analysis of the fluorescence of N-[10(1-pyrenyl)decanoyl]phosphatidylcholine (PyrPC) in multilamellar vesicles of 1,2-dimyristoyl-L-α-phosphatidylcholine (DMPC) shows that i) the components of the membrane mix ideally both in liquid crystalline and in gel phase; ii) the lateral diffusion coefficient of the PyrPC is 25 $\mu m^2 s^{-1}$ in liquid crystalline phase at 30.3°C and 2 $\mu m^2 s^{-1}$ in gel phase at 10°C.

Tu-Pos487

RESOLUTION OF THE ^1L_a AND ^1L_b STATES OF INDOLE IN VAPOR AND JET FROM POLARIZED TWO-PHOTON FLUORESCENCE EXCITATION.

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We have measured the polarized two-photon fluorescence excitation spectrum of indole in both vapor and supersonic jet beams using a pulsed dye laser operating between 530-575 nm, (corresponding to 265-287.5 nm in the UV). The overlapping ^1L_a and ^1L_b bands in this region were resolved by measuring the ratio of fluorescence intensity excited by circularly and linearly polarized light. Experiments on both solutions and molecular orbital theory have established that this polarization ratio for ^1L_b is near 1.5 and that for ^1L_a is near 0.5. The pronounced peaks at 546.0 and 546.7 nm (seen in one-photon vapor spectra at 273.0 and 273.4 nm) in the vapor spectrum 1420 cm^{-1} above the ^1L_b origin are clearly identified as ^1L_a. In the jet, we have assigned lines to ^1L_a ranging from 455 cm^{-1} to 1459 cm^{-1} above the ^1L_b origin. The ^1L_a origin (455-480 cm^{-1} pair) appears to be split by a nearby optically silent ^1L_b state. Previous jet-cooled beam experiments have failed to positively identify any ^1L_a transitions. The developing perception that ^1L_a states are dissociative in vacuum appears to be incorrect.

Tu-Pos488

FLUORESCENCE STUDIES OF *ASCARIS* TRYPSIN INHIBITOR

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Ascaris trypsin inhibitor (ATI) is a single tryptophan protein with five disulfide bonds. ATI has no tyrosine which makes it a valuable model for studying tryptophan photophysics in a protein. At room temperature, the emission max is 337 nm and the steady state anisotropy is 0.257, implying marked immobility of the trp ring but significant solvent exposure. Acrylamide and iodide quenching support these latter inferences. NMR results suggest a highly compact structure but 2D NMR studies were unable to resolve secondary structural features for the entire protein (Agronenborn et al. Biochem. 1990, 29,183-189). CD and FT-IR measurements provide insight into the protein's secondary structure. Despite the apparent rigidity of ATI's tertiary form, the fluorescence intensity decay is extremely complex with as many as four exponential terms. It is not clear whether a discrete lifetime model or a distribution of states provides the best description of the intensity decay profile. A detailed analysis of the fluorescence intensity decay will be presented (Supported by GM 34847)

Tu-Pos489

FLUORESCENCE INTENSITIES AND LIFETIMES OF EUROPIUM-EDTA COMPLEXES,

John Trujillo*, Ray Hapak, Michael T. Henzl and Edward R. Birnbaum, Dept. of Chemistry, New Mexico State University, Las Cruces, NM 88003

A plot of the fluorescence intensity vs. concentration for a titration of the hexadentate ligand, $EDTA^{4-}$ with Eu(III) behaves differently at low vs. high concentrations of EDTA. At low concentrations the fluorescence intensity increases linearly until the 1:1 molar ratio is reached after which there is no further change in intensity with increasing concentration of Eu(III). This is consistent with the formation of a strong 1:1 complex between Eu(III) and EDTA as expected. At high EDTA concentrations, however, the intensity reaches a maximum near a 1:1 molar ratio after which it gradually decreases in intensity. We were interested in this fluorescence behavior since it models the behavior first reported for the titration of the protein parvalbumin with Tb(III) (see Henzl et al., J. Biol. Chem.[1985], 260, 8447 and references therein). Analogous behavior is observed for the terbium ion with EDTA. This quenching behavior at high EDTA concentrations in the presence of excess Eu(III) or Tb(III) is consistent with either a collisional or a static quenching mechanism in which the lanthanide ion in excess of the stoichiometric 1:1 molar ratio quenches the fluorescence of the metal-EDTA complex. Lifetime and intensity measurements as a function of EDTA and Eu(III) concentrations show the mechanism to be primarily collisional in nature, analogous to the parvalbumin case. Unlike the situation for parvalbumin, at pH 6.0 Stern-Volmer plots of the quenching data are not linear. However, linear plots are obtained using the modified Stern-Volmer plot suggesting that the aqueous Eu(III) ion quencher has only limited access to the excited state Eu-EDTA complex. The presence of both the $[Eu(EDTA)(H_2O)_3]^-$ and $[Eu(EDTA)(H_2O)_4]^-$ complexes in solution may be responsible for this behavior in that the collisional quenching rate constant for the two species may be different, resulting in one complex anion being quenched preferentially at a given quencher concentration. This quenching behavior may be a general phenomenon whenever excess Eu (or Tb) ions are present in solution containing a sufficiently high concentration of a Eu (or Tb) protein complex.

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Tu-Pos490

ELECTROSTATIC CONTROL OF ACETYLCHOLINESTERASE CATALYSIS,

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Hydrolysis of Ach^+ by acetylcholinesterase (AchE) occurs with bimolecular rates of $k_{cat}/K_M \approx 10^9$ M^{-1}-min^{-1}, the highest of any enzyme yet studied. One important component underlying this high catalytic efficiency is the presence of a high net negative charge density within the active center. By virtue of the high net charge characteristic of this enzyme, attributable also to the presence of anionic sites topographically remote from the active center, it might be expected that catalysis by AchE would be markedly dependent on the ionic composition of the medium. Yet catalysis is seen to be virtually invariant with ionic strength of the medium (Berman and Leonard, *BIOCHEMISTRY*, 1990), a remarkable observation when considered in light of the sharp ionic strength dependence known for noncovalent ligand association at the active center. In order to examine conformational effects separate from covalent catalysis, we employed as spectral indicators NBD-aminoethyl- and NBD-aminopentyl methylphosphonofluoridates, two structurally-related fluorescent probes that react stoichiometrically at the active center to form stable covalent conjugates, NBD-AE-MP-AchE and NBD-AP-MP-AchE, respectively. The excitation and emission maxima for NBD-AE-MP-AchE appear at 470 and 530 nm, respectively. In the presence of increasing concentrations of NaCl the fluorescence at 530 nm undergoes a 2-fold increase in intensity. The concentration at which the intensity is half-maximal, I_{50}, is approximately 10 mM. Similar results are observed in the presence of K^+. In the presence of increasing concentrations of Mg^{++} the fluorescence intensity undergoes a 50 percent decrease in intensity. The value for I_{50} is approximately 10 μM. Similar results are observed in the presence of Ca^{++}. In all cases these changes occurred without change in spectral position. Of interest is that the effects of the mono- and divalent cations are mutually antagonistic. Taken with corresponding behaviour observed for NBD-AP-MP-AchE, these results indicate that the conformation of AchE as discerned from observation of surface active dipolar fluorescence probes is labile to subtle changes in the concentrations of mono- and divalent cations. The effects for the mono- and divalent ions, since they are opposite in direction and mutually antagonistic, require that the spectral changes arise at least in part from direct ion binding at the protein surface. In view of the invariance of AchE catalysis to changes in ionic strength of the medium, the ion-dependent lability of AchE conformation signifies the operation of a mechanism that is principally electrostatic in nature and that serves to stabilize catalytic efficiency within a narrow range of values.

Tu-Pos491

MATRIX APPROACH TO SOLVING PROBLEMS INVOLVING FLUORESCENCE ENERGY TRANSFER AND DIFFUSION.

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The rate of fluorescence energy transfer depends not only on the distribution of donor-acceptor distances, but also on the relative orientation and lateral or rotational diffusion of the donors and acceptors. Our goal is to extract information on structure (distance/orientation distributions) and dynamics (lateral/rotational motion) from time-resolved data of the vertically and horizontally polarized fluorescence from the donor and from the acceptor. To reach that goal we have designed a mixed analytical-numerical approach:

1. The partial differential equation for distribution of excited donors and its time variation due to decay, transfer and diffusion is transformed into an eigenvalue problem by expanding the distribution in series of functions.
2. The corresponding matrix is infinite. The eigenvalue problem is solved for a truncated NxN matrix and N is increased until convergence is reached.
3. The distribution of excited donors can be obtained similarly.
4. Observables (intensities, anisotropies) are calculated from these distribution functions.

This approach is very effective for solving the Berger-Vanderkooi model [Biophys.Chem.30(1988)257] and related models in which the donor-acceptor distance cannot change but their relative orientation varies due to rotational diffusion. The solution of the truncated 2x2 matrix (which can be obtained analytically) is already a reasonable approximation and in all cases the 5x5 matrix yields the exact solution, which is obtained using a Fortran program on a 386 PC. Results will be shown and discussed. We are working on the application of the method to the case where the donor-acceptor distance changes because of diffusion but reorientations are irrelevant [Biophys.J.55(1989)1225]. This work is supported by a grant-in-aid from the American Heart Association, Kentucky Affiliate, Inc. and by the Lucille P. Markey Foundation (JMB is a L.P.Markey scholar).

Tu-Pos492

MAPPING OF FLUORESCENCE ANISOTROPY IN SINGLE CELLS BY CONFOCAL MICROSCOPY.

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Cytoplasmic and membrane fluidity are important regulators of cell function. Methodology to estimate fluidity with spatial resolution in single living cells has been reported [*Biophys. J.* **57**, 231-241; **57**, 242-254, (1990)]. Fluidity is estimated by measuring the steady-state fluorescence anisotropy (r) of fluorescent probes incorporated into the cytoplasm or membrane. Spatial resolution is obtained by fluorescence microscopy and image analysis. A major limitation of this methodology is that fluorescence from regions of r that are not in the focal plane of the microscope can seriously affect measurement of r in the focal plane. To overcome these limitations, we have used real-time confocal microscopy with Nipkow disk paraxial optics to obtain anisotropy images that reduce almost completely contributions from out-of-focus fluorescence. The method was validated by obtaining confocal anisotropy images of capillary tubes filled with fluorescein in water/glycerol mixtures (r = 0.002 - 0.30). Images of a stack of tubes gave accurate anisotropies; r values were not affected by regions of greatly different r that were not in focus. A tube of r = 0.30 immersed in a sea of fluorescein in water (r = 0.03) gave a 70% elimination of out-of-focus fluorescence, demonstrating the ability to measure r in the presence of intense background fluorescence. Images of BCECF in Swiss 3T3 fibroblasts in two different focal planes revealed different r in nucleoplasm (0.14) and peripheral cytoplasm (0.20), suggesting more binding of BCECF in cytoplasm than in nucleoplasm. Images of rhodamine 123 in mitochondria revealed a uniform r of 0.24 with excellent spatial resolution. The increased resolution afforded by confocal microscopy makes possible the measurement of r in subcellular domains in single living cells with minimal contribution from out-of-focus fluorescence.

Tu-Pos493

A MOLECULAR "LIGHT SWITCH" FOR DNA: $Ru(BPY)_2(DPPZ)^{2+}$.

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Reported is the application of a novel transition metal complex as a sensitive spectroscopic probe for DNA. The complex, $Ru(bpy)_2(dppz)^{2+}$ (bpy = 2,2'-bipyridine, dppz = dipyrido[3,2: a-2'3':c]phenazine, figure 1) shows no detectable photoluminescence in aqueous solution at ambient temperatures, but in the presence of double helical DNA, to which the complex binds avidly, intense photoluminescence is observed. Time correlated single photon counting experiments of $Ru(bpy)_2(dppz)^{2+}$ in the presence of 100 μM poly d(GC)•d(GC), at 25 °C reveal a biexponential decay of the emission with a short lived component of 75 ns (64 %) and a longer lived component of 256 ns (36 %). The photoluminescence of $Ru(bpy)_2(dppz)^{2+}$ is strongly dependent upon the helical structure of DNA; with B-form poly d(GC)•d(GC), emission is centered at 628 nm (λ_{ex} = 482 nm) while in the presence of Z-form poly d(GC)•d(GC) the relative intensity of the steady state luminescence is even greater than that found with B-DNA, and the emission maximum is shifted to 640 nm. This intense luminescence observed with DNA is comparable in intensity to that found for the complex in nonaqueous solutions such as in isopropanol ($\Phi \geq 0.02$) in the absence of DNA. Interestingly, only weak emission is observed in the presence of A-form poly r(AU)•r(AU) with emission centered at 650 nm. The enhancement factor in aqueous solution for photoluminescence of $Ru(bpy)_2(dppz)^{2+}$ in the presence of DNA is estimated to be > 10^4. The complex's usefulness as a sensitive, non-radiactive, luminescent DNA probe in both heterogenous and homogenous assays will be described.

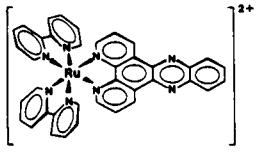

Figure 1

Tu-Pos494

PHOSPHORESCENCE AND OPTICALLY DETECTED MAGNETIC RESONANCE (ODMR) STUDIES OF THE INTERACTION BETWEEN MELITTIN AND BEE VENOM PHOSPHOLIPASE A_2 (PA_2) IN DYMYRISTOYL PHOSPHATIDYLCHOLINE (DMPC) VESICLES

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The polypeptide melittin has a synergistic effect on the activity of bee venom phospholipase A_2 (PA_2) in the presence of phospholipids. This interaction has been studied through the photo-excited triplet states of the tryptophans (Trp) in PA_2 (2 Trps) and melittin (1 Trp) in DMPC vesicles using phosphorescence and ODMR spectroscopy.

The phosphorescence spectra show that the complex of PA_2 and melittin combined in DMPC vesicles produces a red-shifted emission maximum ($\lambda_{0,0}$ = 414.3 nm) compared with either melittin in DMPC ($\lambda_{0,0}$ = 412.5 nm) or PA_2 in DMPC ($\lambda_{0,0}$ = 407.5 nm). This evidence, combined with measurements of the variation of the zero-field splittings (ZFS) with emission wavelength suggest the presence of triplet state heterogeneity corresponding to Trp in different environments. The effect of stoichiometry on the interaction between PA_2 and melittin in DMPC vesicles will be addressed further on the basis of changes in the ZFS parameters D and E.

Tu-Pos495

SPECTRAL DIFFERENTIATION OF INTRINSIC PROTEIN TRYPTOPHAN QUENCHING BY DISULFIDES AND CHARGED RESIDUES.

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Intramolecular quenching of protein tryptophan fluorescence may be attributed to proximal disulfide bonds and/or charged groups. The diversity of quenching groups, *vis-a-vis* their electronic structure and orientation with respect to a fluorophore, suggests that various mechanisms may fall under the aegis of "static" quenching. One may posit distinctions between the quenching that arises from stable charge-transfer complexes and that which ensues when energy acceptors are locked in the proximity of a fluorophore by virtue of protein tertiary structure. Fluorescence emission studies can quantitate the extent of quenching but cannot elucidate the mechanisms of non-radiative energy dissipation. However, perturbations observed in absorbance spectra may represent interactions of the transition dipole with its environment. Such interactions are dependent upon distance and orientation factors and thus can suggest specific tertiary structural relationships.

Select single tryptophan proteins of known structure, including Streptomyces griseus protease A, azurin, eel troponin C, and mutants of E. coli thioredoxin were used to evaluate the two types of quenchers of interest. Spectral data will be presented which supports distinguishing characteristics between the tryptophan fluorescence quenching modes of disulfides and charged amino acid residues within a protein. (Supported by GM 34847.)

Tu-Pos496

MELANIN AND CATIONIC PORPHYRIN INTERACTION. OPTICAL ABSORPTION AND FLUORESCENCE STUDIES.

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The interactions between synthetic melanin (obtained from autooxidation of L-hydroxy phenylalanine) and cationic porphyrins were studied using optical absorption and static and time-resolved fluorescence spectroscopy. The porphyrins investigated were tetra-meso substituted pyridyl porphyrins: TMPyP (tetramethylpyridylporphyrin), TBzPyP (tetrabenzopyridylporphyrin) and their metallic forms, ZnTMPyP, and ZnTBzPyP. Another porphyrin used was ZnTAlPyP (Zinc tetraallylpyridylporphyrin). It was shown that melanin interacts with all these porphyrins changing their electronic structures. The fluorescence of the porphyrins were strongly quenched by melanin. Steady state and time resolved fluorescence results demonstrated that there was formation of non-fluorescent melanin-porphyrin complexes. The complication is due to ionic type interactions and it is estimated that about 5 molecules of porphyrin interact with one molecule of melanin. The evidence indicates that the pigment melanin dissipates the energy of the electronically excited states through non radiative processes. The results indicate the importance of the physico-chemical environment of the melanin in the cellular processes in which it is involved.

Financial support: FAPESP and USP-BID agreement.

Tu-Pos497

RAMAN SPECTROSCOPY OF A CHROMOPHORIC ACYLENZYME OF SUBTILISIN-BPN' AND ITS OXYANION HOLE MUTANT, N155G

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Unlike α-chymotrypsin, the bacterial serine protease, subtilisin-BPN' uses an asparagine residue (N155) to form one of the two oxyanion intermediate stabilizing H-bonds known as the oxyanion hole. Wells, and co-workers have produced a series of N155 mutations [Wells, J.A., et al., (1986) *Phil. Trans. R. Soc. Lond. A 317*, 415-423]. We have sought to directly measure the interaction of the oxyanion hole H-bonds with an acylenzyme intermediate by monitoring changes in its Raman spectrum due to the N155G mutation. Acylenzymes are made by selectively acylating the catalytic serine of wild-type(WT) and N155G subtilisin-BPN' with dimethylamino-benzoyl(DAB)-imidazole. The resulting DAB-WT and DAB-N155G acylenzymes deacylate catalytically, yet slowly ($k = 10^{-4} s^{-1}$ @ pH8), DAB-WT deacylating 40-fold faster than DAB-N155G and 150-fold faster than DAB-α-chymotrypsin. The DAB-WT and DAB-N155G Raman spectra show a number of differences; of greatest interest is the stretching vibration of the carbonyl group adjacent to the scissile serine ester bond - this vibration is extremely sensitive to the solvent environment of the carbonyl oxygen atom. The spectra reveal at least two distinct carbonyl populations in both DAB-WT and DAB-N155G at pH4. Based on comparison to spectra taken in D_2O, we have assigned two acylenzyme conformations: the carbonyl oxygen positioned such that it is polarized by the oxyanion hole; and the oxygen H-bonded to a water molecule as has been observed in the crystal structures of various acyl-chymotrypsins. The latter conformation is not affected by the mutation, whereas, the former is significantly diminished in DAB-N155G.

Tu-Pos498

VIBRATIONAL CICULAR DICHROISM OF THE AMIDE II BAND IN SOME MODEL POLYAMINO ACIDS AND PROTEINS IN WATER

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We have measured the Vibrational Circular Dichroism (VCD) spectra of poly-L-glutamic acid, poly-L-lysine, hemoglobin, myoglobin, albumin, α-chymotrypsin, carbonic anhydrase, ribonuclease-S and ribonuclease-A in water. In addition, characteristic changes have been observed in the amide II VCD due to helix - coil and helix - sheet transitions in model systems such as poly-L-glutamic acid and poly-L-lysine. The amide II VCD for the predominantly helical proteins is the same as that of helical poly-L-glutamic acid, a negative band lying to the low energy side of the absorbance maximum. The β-sheet and coil conformation spectra are less well defined. Our peliminary measurements indicate that conformational distributions in proteins can be characterized using VCD of the amide II band of proteins in water as well as the previously studied amide I' bands in D_2O.

Tu-Pos499

TRANSIENT RESONANCE RAMAN STUDIES OF NiPPIX EXCITED STATES IN MICELLAR SYSTEMS.

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In this study we report on the photodynamic behavior of Ni protoporphyrin IX in both CPB(cetylpyridinium bromide) and AOT(bis(2-ethylhexyl) sulfosuccinate sodium salt) micellar systems as studied by transient resonance Raman spectroscopy. The reversible formation of NiPP excited states was observed in both of these solvent systems by using transient resonance Raman spectroscopy. The transient spectra taken employed pulsewidths of either 35 picoseconds or 10 nanoseconds. The effect of the micellar environment on the Ni centered (d-d) excited states was observed by comparing transient Raman spectra taken at low and high photon densities. These studies corroborate and expand upon results of an earlier study[1] which indicated that the excited state electronic and vibrational properties of the porphyrin are very sensitive to the porphyrin environment. A correlation between the solvent sensitive Ni centered (d-d) excited state and the solvent parameter ET(30) was investigated for a number of micellar systems. The ET(30) probe has been used as an effective measure of the polarity of the hydrocarbon/water interface in micellar systems.

[1] Findsen, E. W., Shelnutt, J. A., Ondrias, M. R. J. Phys. Chem., 1988, 92, 307-314.

Tu-Pos500

CIRCULAR DICHROISM STUDY OF THE EFFECT OF CADMIUM ON CONFORMATION OF MEMBRANE PROTEINS OF HUMAN ERYTHROCYTES AND THE AMELIORATIVE EFFECT OF SELENIUM, ZINC AND DTT IN VITRO.

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Circular Dichroism(CD) spectrum was used to investigate the effects of cadmium on the spatial structure of human erythrocyte membrane proteins in vitro, and the antagonistic effects of zinc, selenium and dithiothreit(DTT) against cadmium were also studied. Cadmum at the levels of 10, 40, 160, 640 and 960 μM could induce the changes of membran protein conformation of erythrocyte ghosts, including decreases of α-helix and β-helix percentages and increase of γ-curl percentage, showing dose and time dependent manners. Simultaneously with cadmium exposure, adding DTT(1 mM) and selenium(2 μM) could completely and partly prevent the effects of cadmium on protein conformation respectively, the antagonistic effect of zinc was not observered, however, in this study. Since DTT is a thiol reducing agent and could protect the protein thiol effectively, the results of this study indicate that cadmium may induce the changes of membrane protein conformation mainly by affecting the membrane protein thiol groups.

Tu-Pos501

MONITORING CHEMICAL MODIFICATION OF HISTIDINE RESIDUES IN PROTEINS BY RAMAN DIFFERENCE SPECTRSCOPY

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We have shown that Raman difference spectroscopy can follow the titration of histidines in transferrin as well as in solution (M. Lee, et al., *Biophys. J.*, (1990) 57 51a). We have now applied Raman difference spectroscopy to study the modification of histidine residues in proteins by diethylpyrocarbonate (DEP). DEP reacts specifically with the histidyl residues in proteins between pH 5.5 and 7.5 to yield an N-carbethoxyhistidyl derivative. This reaction can be followed spectrophotometrically by the increase in absorbance at 240nm. However, absorption peaks are broad and sometimes nonspecific. A more direct confirmation of the modification is desirable. Raman difference spectroscopy provides a very specific method to determine the accessibility of histidine residues in proteins.

We have measured the modified histidine residues in transferrin as well as in solution at various pH values. This method can potentially be used to study modifications of other amino acid groups in proteins.

Tu-Pos502

EXAFS ANALYSIS OF VITAMIN B_{12} AND RELATED COBALAMINS. *I. Sagi and M. R. Chance. Georgetown University, Department of Chemistry, Washington D.C. 20057.*

The B_{12} cobalamins and coenzymes are essential to metabolism and are known to participate in a wide range of catalytic reactions. A characteristic step in all these reactions is cobalt-carbon bond cleavage that can be homolytic or heterolytic depending upon the enzyme. Structural analysis of cobalamins is a well studied field, however a number of structures important to nutrition and catalysis are not well understood. Accurate crystal structures are available for adenosylcobalamin (Savage, et. al., *Acta Cryst.*, **B43**, 1987, p. 296.) and methylcobalamin (Rossi et. al., *J. Am. Chem. Soc.*, **107**, 1985, p. 1729.). Although aquocobalamin, base-off cobalamin and cyanocobalamin are important, crystallographic structures are either unavailable or unreliable. In order to provide more accurate structures we have carried out EXAFS studies of these cobalamin compounds. The EXAFS solution structure and the x-ray crystallographic structures of adenosylcobalamin and methylcobalamin closely agree, suggesting that crystal packing does not significantly perturb the cobalt-ligand distances for these species. The Co-N average bond distances in the equatorial plane for these compounds are 1.90±.01 Å, while the Co-N and the Co-C axial bond distances are 2.20±.02 Å and 2.00±.02 Å respectively. The EXAFS solution for cyanocobalamin is much more accurate than the x-ray crystallographic solution (Brink-Shoemaker, et. al., *Proc. R. Soc. London.*, **278**, 1964, p. 1.) and demonstrates an average Co-N equatorial distance at 1.89±.01 Å, a Co-N axial distance at 2.15±.03 Å and a Co-C axial ligand distance (to cyanide) of 1.89±.06 Å. The EXAFS analysis suggests that for the compounds observed so far the equatorial effects are small, while the axial changes are substantial. We will present the EXAFS solutions for aquocobalamin and base-off cobalamin and compare them to other cobalamins structures.

This research is supported by CSRS U.S. Department of Agriculture under grant #90-37200-5357 of the Program in Human Nutrition.

Tu-Pos503

ABNORMAL MOLECULAR VIBRATIONS IN VARIOUS HUMAN CANCERS: PRESSURE-TUNING INFRARED SPECTROSCOPY. **P.T.T. Wong* and B. Rigas†. *Steacie Inst. Molecular Science, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6, †Department of Medicine, Cornell University Medical College, New York, N.Y. 10021.**

Fourier-transform infrared spectroscopy (FT-IR) has been developed for the study of the composition and structural properties of cellular components within intact tissues in our Ottawa laboratory. Using FT-IR spectroscopy in combination with high-pressure (pressure-tuning infrared spectroscopy), we observed that human cancers displayed abnormal infrared spectra at both atmospheric pressure and high pressures compared to corresponding normal tissues. These changes involving the whole frequency region of internal vibrations, in particular the phosphate and the C-O stretching modes, the CH stretching and bending regions and the C=O stretching modes, reflect alteration in the structure of important informational and structural molecules in the malignant tissues. Cultured human cell lines displayed similarly abnormal infrared spectra. Some spectral changes are common to various human and animal cancers.

Tu-Pos504

WATER-PEPTIDE CARBOXYL HYDROGEN BONDS MAY BE VERY REPULSIVE AT SHORT DISTANCES. Wilson Radding, Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294.

Circular dichroism (CD) spectra of alanyl-alanine diketopiperazine [c-(L-ala)$_2$] in water, methanol and trimethylphosphate (TMP) at room temperature or water-glycerol, methanol-glycerol and TMP at 77°K allow one to construct a diagram of relative energy levels of the n_p, σ^*, and π^* orbitals in different environments. From this diagram it is possible to obtain information about the hydrogen bond repulsive potential by using algebra and the following assumptions: 1) The π^* orbital is not sensitive to solvent at 298°k. 2) The n_p orbital is shielded from temperature lowering induced solvent compression effects in TMP. 3) The σ^* orbital energy is dependent on local repulsive effects. 4) Only the repulsive part of the potential need be considered. 5) Solvent compression is isotropic and can be measured as a global property. The results indicate that when the hydrogen bond is compressed below its equilibrium length, the water-peptide carboxyl hydrogen bond potential function is considerably steeper than the methanol-peptide carboxyl one. In fact, by this estimate the water-peptide carboxyl hydrogen bond has a steeper potential function in its repulsive region than theoretical hydrogen bond potential functions. This is in accord with statistical compilations of hydrogen bond lengths which indicate there is an abrupt cut-off of water-peptide carboxyl hydrogen bonds below 2.7°A [Mitra, J. and Ramakrishnan, C. Int. J. Pept and Prot. Res. 9,27(1977)]. It seems possible that extreme incompressibility might make a water-peptide carboxyl hydrogen bond an ideal fulcrum for concerted rearrangement in large proteins.

Tu-Pos505

PROPERTIES OF THE HYDRATION LAYER OF DNA STUDIED USING MICROWAVE ABSORPTION SPECTROSCOPY, H.R.Garner, T. Ohkawa, O. Tuason, R. L. Lee, Institute for Development of Advanced Technologies, General Atomics, P.O. Box 85608, San Diego, CA 92186, (619) 455-3464. The imaginary part of the dielectric constant for the hydration layer of microvolume samples (2 uL at 0.05 kg/L) of Calf Thymus DNA has been measured in a swept manner over the frequency range of 2 to 26.5 GHz. The relaxation time for the hydration layer of DNA at 26°C was determined to be 0.015 ns, an upshift from that of free water, 0.0087 ns. The hydration layer volume as determined from the absorption magnitude is small, approximately 0.66 that of the DNA volume, or about 1 water molecule thick. A temperature controlled sample holder has enabled measurements of double stranded DNA as well as thermally denatured DNA. A marked change in the microwave absorption attributable to the hydration layer is seen at the denaturation temperature of the DNA indicating that the bound water is converted to free water.

Tu-Pos506

CHEMILUMINESCENCE OF ETYA,THE ALKYNE ISOMORPH OF ARACHIDONIC ACID EXCEEDS THAT OF THE PARENT COMPOUND. Anderson,K.M.(1,2),Harris,J.E.(1),Dept. Med (1) and Biochem.(2),Rush Med. Col.,Chigago, Il. ETYA (5,8,11,14-eicosatetraynoic acid),the isomorph of arachidonic acid(5,8,11,14-eicosatetraenoic acie,AA) inhibits stimulated chemiluminescence (CL) and is itself chemiluminescent. Because ETYA induced ultrastructural findings consistent with "oxidative stress", generally ascribed to excess free radicals (FR), and reduced the number of mitochondria in human prostate PC3 cells, we measured ETYA-induced CL by liquid scintillation spectrometry in the out-of-coincidence mode at R.T. with the following preliminary results: ETYA in DMSO at -20° gradually turned yellow (autooxidation);(2) ETYA in DMSO exhibited CL, which increased after mixing (0.1% final conc.) with 1 ml buffer. This increase was prevented by N_2 in the counting vial, (which O_2-dependent event seems to exclude ETYA as a superior "fluor" participating in a photochemical event);(3)NDGA (nordihydroguaiaretic acid) inhibited CL; (4) Luminol, but not lucigenin could augment ETYA CL;(5) Cells could increase ETYA-CL in excess of that due to ETYA alone;(6)AA did not duplicate these results. Conclusions: Although CL need not entail a FR mechanism, PUFA autooxidation and these initial results, coupled with the ultrastructural findings in PC3 cells are consistent with the generation of ETYA-FR, augmented by cellular metabolism.Putative ETYA-FR could contribute to the ultrastructural changes in PC3 cells, that were absent in U937 monoblastoid cells with their likely greater concentration of antioxidant defences. Destruction of PC3 mitochondria and the generation of CL in this complex system may certainly involve additional mechanisms, and direct evidence for ETYA-induced FR and/or specific ETYA or cell membrane autooxidation products is required to support this hypothesis.

Tu-Pos507

CHARACTERIZATION OF THE INTERACTION OF THE RADIOPROTECTANT 1-METHYL-2-[2-(METHYLTHIO)-2-PIPERIDINOVINYL]-QUINOLINIUM IODIDE WITH SUPERCOILED DNA. C. E. Swenberg, Radiation Biochemistry Department, Armed Forces Radiobiology Research Institute, Bethesda, MD 20889-5145, S. Birke and N. E. Geacintov, Chemistry Department and Radiation and Solid State Laboratory, New York University, New York, NY 10003.

The interaction of the radiation protectant chemical 1-methyl-2-[2-(methylthio)-2-piperidinovinyl]-quinolinium iodide (VQ) with linear and supercoiled pIBI30 DNA was studied by flow linear dichroism spectroscopy, equilibrium dialysis, circular dichroism, and UV absorption spectroscopy. The negative linear dichroism spectra of VQ-DNA complexes throughout the 220-500 nm wavelength region, a red shift in the VQ main absorption band (at 452 nm) of 1-2 nm upon binding to DNA, and a concentration-dependent unwinding of supercoiled DNA suggest that the primary mode of interaction of VQ with DNA (at least at low concentrations) is intercalative in nature. A least-square analysis of the equilibrium dialysis binding of VQ to supercoiled DNA using the McGhee-von Hippel equation gives an association constant K = 7300 ± 300 M^{-1} and an exclusion number n in the range of 3.3-5.3. The lower value is obtained when polyelectrolyte effects are also taken into account. Because quinolinium iodide derivatives with different substituents and DNA binding affinities can be synthesized, this family of compounds could be employed to probe relationships, if any, between radioprotective efficacy and DNA binding affinity.

Tu-Pos508

Ultraviolet Resonance Raman Spectroscopy of the X-Pro Imide Bond

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Ultraviolet Resonance Raman (UVRR) spectroscopy is used to selectively study vibrations of the X-Pro imide bond in proteins. The structure of this bond, which lacks the usual amide hydrogen, bestows unique absorption and vibrational properties. Tuning the incident laser light to 210 nm, redshifted from the usual amide absorption, permits selective observation of the imide vibrations via the resonance Raman effect. The imide bond vibration at around 1450 cm^{-1} is distinct from its amide counterpart. The spectra show two peaks in this region whose populations change upon protein denaturation, reflecting different influences from the proline environment. UVRR data for model peptides are presented which demonstrate a sensitivity of the X-Pro vibrational frequency on hydrogen bonding, and which are used to interpret the protein results. We conclude that UVRR spectroscopy can provide selective information on the structural characteristics of proline in proteins, and present results from representative examples such as ribonuclease, cytochrome c, and α-lactalbumin.

Tu-Pos509

NEAR-INFRARED RAMAN SPECTROSCOPY OF PROTEINS

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Many biological compounds have eluded conventional Raman spectroscopy relying on excitation in the visible, because they either exhibit fluorescence or degrade under the laser beam. A promising alternative is to use excitation in the near-infrared, which is provided by a Nd:YAG (1064 *nm*) or a tunable Ti:sapphire laser (700 - 1000 *nm*). Raman spectra in the absence of fluorescence and without resonance enhancement can be obtained and provide specific information on molecular conformation both in the solid state [1] and in solution. The application of this technique to strongly absorbing and photolabile proteins such as bacteriorhodopsin will be demonstrated and the results compared with those using visible excitation.

[1] C. G. Zimba, V. M. Hallmark, J. D. Swalen, J. F. Rabolt, Appl. Spectr. **41**, 721 (1987)

Tu-Pos510

EFFECTS OF NON-SPHERICALLY SYMMETRIC GEOMETRIES ON DISTANCE DETERMINATION BY DIFFUSION ENHANCED ENERGY TRANSFER J.V. Mersol, A. Gafni, D.G. Steel (Intro by Raoul Kopelman) The University of Michigan Ann Abor, MI 48109

Protein luminescence quenching by diffusion enhanced energy transfer is a useful technique for distance determinations. A potentially serious limitation of this technique is the extent to which the models used actually represent the configuration and dynamics of proteins in solution. Most representations of energy transfer at the rapid diffusion limit, where the donor and acceptor are free in solution, assume that the orientation angles in the dipole-dipole interaction term are completely randomized due to rotation of the molecules involved. This assumption, however, is not true for structures in which there is not complete spherical symmetry around the chromophores. In this study the theory is extended to account for the incomplete angular averaging in several such models. For transfer in a geometry involving spherical molecules, the lack of complete averaging is small when the chromophores are not placed near the surface. For geometries involving a planar surface (e.g., a membrane), however, the quenching rate can vary over a factor of two depending on the orientation of the chromophore in the plane. These improvements in the theory are then applied to experimental results obtained using the phosphorescent tryptophan of proteins under different conditions as energy donors to freely diffusing energy acceptors.

Tu-Pos511

APPLICATION OF FTIR-PAS, -ATR, AND -DRIFT TO THE DETECTION OF MICROBIALLY-INFECTED CORN, *Richard V. Greene and Sherald H. Gordon, Biopolymer Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Northern Regional Research Center, 1815 N. University St., Peoria, IL 61604*

Evaluation of agricultural grains, such as corn, suffers from a lack of suitable techniques which can analyze solid-state samples. Three techniques, photoacoustic spectroscopy (PAS), attenuated total reflectance spectroscopy (ATR) and diffuse reflectance spectroscopy (DRIFT), can be integrated with Fourier transform infrared (FTIR) spectrometers to yield IR spectra of solid materials. The relative signal-to-noise ratios of the three techniques, determined by the root mean square of the differences observed in consecutive FTIR spectra of corn, were 450 for PAS, 1200 for ATR and 6600 for DRIFT. Dramatic differences were observed between spectra generated from corn which had been field-infected with *Fusarium* sp., a fungus known to produce several toxic secondary metabolites, and uninfected corn. This allowed for detection of the microbe at the 1% level. Similar results were observed for *Aspergilli*, which produce aflatoxin as well as other toxic compounds. Interestingly, PAS was the most sensitive technique for such determinations as most of the infection appeared to be located at the surface of the kernel.

Tu-Pos512

THE IRON IN LIPOXYGENASE BY MOSSBAUER SPECTROMETRY. R.H. Sands, R.T. Carroll, J.F. Thompson, W.R. Dunham and M.O. Funk, Jr., Biophysics Research Division, The University of Michigan; Agricultural Research Service, USDA, Ithaca, NY, and Department of Chemistry, University of Toledo, OH.

Incorporation of ^{57}Fe into two lipoxygenase isoenzymes from soybeans permitted the use of Mossbauer spectrometry, which showed high-spin Fe(II) in the native enzyme. From model compounds and the sign of the electric field gradients, the most likely environment of the iron is oxygen and nitrogen ligands in approximately octahedral coordination. Both enriched and natural abundance native enzymes had the same high-spin Fe(II) Mossbauer parameters confirming that the iron environments in the enzymes isolated from cultured seeds and dry soybeans are the same. The spectra (4.2-25OK) after oxidation of the iron by the product of lipoxygenase catalysis are extremely broad (~20mm/s), as were the EPR signals, indicating paramagnetic broadening even at the relatively high temperatures. Treatment of the product oxidized enzyme with linoleic acid (substrate) under anaerobic conditions produces Fe(II) Mossbauer signals, indicating that lipoxygenase iron cycles between the ferric and ferrous states during catalysis.

The compound Fe^{2+}DTPA is proposed by us to be a likely model for the active site of lipoxygenase. The x-ray crystallographic structures of two forms of this compound have been determined. The iron DTPA salts share many spectroscopic properties in common with lipoxygenase that have not appeared in proteins that are not functional dioxygenases.

Tu-Pos513

MÖSSBAUER AND EPR CHARACTERIZATION OF NITRITE REDUCTASE FROM *Desulfovbrio desulfuricans*

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Nitrite reductase isolated from *Desulfovbrio desulfuricans* (ATCC27774) is a membrane-bound multi-heme cytochrome (M_r=66,000) which catalyzes the 6-electron reduction of nitrite to ammonia. Mössbauer and EPR spectroscopy are used to study this enzyme. Our Mössbauer measurements clearly indicate that this heme protein contains six distinct heme groups:one high-spin(S=5/2) and five low-spin(S=1/2) ferric hemes. Characteristic hyperfine parameters for all six hemes are obtained. At pH 7.6, the EPR spectrum of the native enzyme is very complicated in which only one typical low-spin ferric heme signal at g=2.96, 2.28 and 1.50 is detected, plus some broad resonances are also observed. The broad resonances are attributed to heme-heme interactions in the enzyme. The presence of heme-heme interactions is clearly demonstrated by the low temperature (4.2 K) Mössbauer spectra which reveal that four out of six hemes, including the high-spin heme, are magnetically coupled. The enzyme can be fully reduced by dithionite or hydrogenase under H_2 atmosphere. Reaction of nitrite with the fully reduced enzyme reoxidizes all the low-spin hemes. The high-spin heme, however, is found to complex with NO, which suggests that the high-spin heme may be the substrate binding site and that NO may be an intermediate present in an enzyme-bound form.