Protein Structure and Function

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PROTEIN CATABOLISM IN BEAN LEAF DISCS: EFFECTS OF LIGHT AND OXYGEN

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A net protein degradation can be initiated by the excision of segments or discs from mature leaves. The level of proteins and their breakdown products was found to be affected by environmental conditions. In these experiments, discs from mature, non-senescing primary leaves of beans were incubated under various conditions and analysed by colorimetric procedures, SDS-PAGE and immunoblotting. The light harvesting chlorophyll binding protein (LHCP; major protein in thylakoids) disappeared more rapidly in the light than in the dark. In contrast, ribulose-1,5-bisphosphate carboxylase (Rubisco; major stromal protein) was more rapidly degraded in the dark. The presence or absence of oxygen was more important for the catabolism of Rubisco than for LHCP degradation. In the dark, under nitrogen a polypeptide of 45 kD accumulated. This polypeptide was recognized by antibodies against Rubisco and it appears likely that it derived from proteolysis of the large subunit of Rubisco. Our results indicate, that stroma (Rubisco) and thylakoid (LHCP) proteins in intact cells undergo differential net degradation dependent upon illumination conditions and oxygen availability.

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PRECURSOR PRODUCT RELATIONSHIP IN *ALLOMYCES ARBUSCULA* CALPAIN-LIKE PROTEASE

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Calpain-like protease was reported as a homodimer of 40 kD peptide (Ojha and Wallace, 1988). Subsequently it was found that a 43 kD protein also copurify with the major 40 kD active peptide. It was postulated that the autoproteolysis of 43 kD peptide generated 40 kD fragment (Ojha, 1990; Ojha and Favre, 1990, in press). Polyclonal antibodies raised against 43 and 40 kD peptides show approximately 50% cross reactivity. Immunoblots with 43 kD antibody of proteins extracted under harsh denaturating conditions and separated by mono- and high resolution bi-dimensional gel electrophoresis show a single, approximately 80 kD peptide. It was concluded that 80 kD is the presursor protein which after processing generates the active 43 and 40 kD peptides.

Ojha, M. and Wallace, C.J.A. 1988. J. Bacteriol. 170:1254.
Ojha, M. 1990. *In* Calcium as an Intracellular Messenger in Eucaryotic Microbes, ASM, Washington, pp. 192.

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ACTIN IN THE CILIATED PROTOZOAN *Climacostomum virens*: PURIFICATION BY DNase I AFFINITY CHROMATOGRAPHY, FILAMENT FORMATION AND BIDIMENSIONNAL GEL CHARACTERIZATION.

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A monoclonal anti-actin antibody (LIN,1981) labels a 43,5 kD band on western blots of *Climacostomum* cells extracted according to Pollard or Zechel. That band does not react with an anti-asmooth muscle actin mab (SKALLI et al.,1986) nor with an anti-asarcomeric actin mab (SKALLI et al.,1988). This protein binds to DNase I and can be extracted and purified by DNase I affinity chromatography. After addition of ATP, Mg and/or KCl, the extracted protein precipitates and forms filaments which are observed in electron microscopy after negative staining. Two-dimensionnal gel analysis reveals that purified *Climacostomum* actin focuses as three spots which are more basic than the mammalian actin isoforms.

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BIOGENESIS OF MUSCLE CYTOARCHITECTURE: INTRA-COMPARTMENTAL SORTING OF CONTRACTILE ISOPROTEINS.

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During myogenesis, the intra-compartmental sorting of the myosin light chain MLC 1f was investigated by a epitope tagging technique. Sarcomeric MLC 1f does not associate well with the non sarcomeric cytoskeleton, however, binds preferentially to the sarcomeric MHC in embryonic cardiomyocytes. Detailed analysis of specific MLC 1f binding to sarcomeres was investigated in adult rat cardiomyocytes, where a stress fiber-like cytoskeleton coexists with myofibrils. Upon transfer of tagged MLC 1f cDNA by microinjection, the resulting MLC1f protein was bound almost exclusively to the sarcomeric MHC. Analysis by confocal laser scanning microscopy showed that sarcomer formation was not uniform throughout the cells and several patterns of myofibrillar association of the newly synthesized proteins were found. Deletion mutants and chimeric constructs of sarcomeric and nonsarcomeric MLC showed that there are three functionally distinct domains in the MLC. While the N-terminal domain seems not to be associated with the sorting, the C-terminal third appears to be responsible for the basal MHC binding and the middle third might modulate the preferential interaction with different MHC isoproteins. Studies on the importance of distinct amino acids for the sorting process are in progress. First studies with actin isoproteins indicate that α-smooth actin preferentially sorts to non sarcomeric sites, while sarcomeric isoforms like α-sarcomeric actins or α-myosin heavy chains, appear to sort to the myofibrillar organelles.

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CHARACTERIZATION OF TUBULIN ISOFORMS FROM THE ZOOSPORES AND MYCELIA OF *ALLOMYCES ARBUSCULA*

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We have characterized the tubulin isotypes of *Allomyces arbuscula* in order to understand their function during growth and differentiation. Immunoblots of one dimensional gel electrophoresis of total proteins extracted from zoospores and mycelia (12 h growth) showed two tubulin bands (α, Mr 55000 and β, Mr 52000) when revealed by the mAbs (Amersham N° 356, 357) against α and β-tubulin. Two-dimensional gel electrophoresis of the same extract and immunoblotting with mAbs demonstrated the presence of only one α and one β-tubulin polypeptides with pI=5.6-6. However, if these gels are silver-stained, two α-tubulin spots are revealed which could correspond to the α_1 and α_3 isoforms described in *Chlamydomonas* by L'Hernault and Rosenbaum (1985). Our α_3 spot might be acetylated as also found by Aliaga and Pommerville (1990). Such an acetylated isoform could play a role in the stability of microtubules especially requested for the flagellated zoospores.

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TUBULINS FROM FIVE SPECIES OF CELLULAR SLIME MOULDS: ANALYSIS BY SDS-PAGE AND IMMUNOBLOTTING

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We analyzed Triton X-100 extracts from vegetative amoebae of the five species of cellular slime moulds *Dictyostelium discoideum, Polysphondylium violaceum, Acytostelium leptosomum, Protostelium mycophaga*, and *Acrasis rosea*. The monoclonal antibodies TAT-1 (Sherwin & Gull, 1989), specific for α-tubulin, and Tu-06 (Draber et al. 1989), specific for an N-terminal determinant of ß-tubulin, clearly labelled the subunits of twice-cycled pig brain tubulin. The M_r was 57 k for α-tubulin and 52 k for ß-tubulin. Except for *A. rosea*, TAT-1 also reacted with a single polypeptide on immunoblots of the slime mould extracts. The M_r of these polypeptides were similar to the M_r of pig brain ß-tubulin, i.e. 53 k for *D. discoideum* and *P. violaceum*, and 51 k for *A. leptosomum* and *P. mycophaga*. Tu-06 revealed a more complex pattern of polypeptides with an M_r higher or lower than either subunit of the brain tubulin control. In *D. discoideum*, a polypeptide of ca. 51 k was detected by Tu-06, but an additional polypeptide of higher M_r was labelled in the pellet of the extract. In extracts from *P. violaceum, A. leptosomum*, and *P. mycophaga* Tu-06 reacted with a polypeptide that migrated more slowly than the corresponding α-tubulins, but in extracts from *A. leptosomum* and *P. mycophaga* additional polypeptides of lower M_r were also present. We now address questions about the nature of the polypeptides reacting with Tu-06 and explore post-translational modifications of the α-tubulins.

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CAP-23, A CORTICAL CYTOSKELETON-ASSOCIATED PROTEIN OF DEVELOPING TISSUES AND NEURONS WITH PROPERTIES SIMILAR TO THOSE OF GAP-43

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We have searched for proteins with physical properties and subcellular location similar to those of GAP-43, a neuron specific protein, whose expression pattern and subcellular distribution indicate that it might play an important role in axonal growth. We report here on the identification, primary structure and distribution of one such protein, CAP-23, a novel cortical cytoskeleton-associated protein identified in chick and rat. Properties shared by CAP-23 and GAP-43 include high hydrophilicity and an unusual amino acid composition, phosphorylation by PKC, comparable abundancy in the developing nervous system and a characteristic granular immunofluorescence labeling pattern in neuronal growth cones, which for CAP-23 was also seen in cultured nonneuronal cells. CAP-23 was a ubiquitous protein in E-2 chick embryos, that subsequently became restricted to an increasingly narrow range of tissues and cell types. In the nervous system, were CAP-23 was not restricted to neurons, its levels varied regionally and quantitatively during development. CAP-23 and GAP-43 might be small, highly hydrophilic components of the subplasmalemmal compartment, whose presence is linked to specialized cellular functions.

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EXPRESSION OF PRESYNAPTIC AND CYTOSKELETAL PROTEINS AFTER BLOCKADE OF ACTIVITY AND EARLY TARGET REMOVAL IN THE CHICK EMBRYO.

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Our aim is to determine whether, during neuronal development, the expression of presynaptic proteins follows an endogenous programme of differentiation or results from cell-cell interactions. Using immunocytochemistry, we have determined that the two presynaptic proteins, SNAP-25 and synaptophysin, are expressed late during development, at the time of synaptogenesis, in both the chick retina and neural tube. Early removal of the limb bud had no effect on the expression of both proteins by motor neurons. In contrast, blockade of electrical activity, before synaptogenesis, by intraocular TTX injections, strongly reduced SNAP-25 and synaptophysin expression in the retina.

Taken together, these data suggest that increase in electrical activity during synaptogenesis play an important role in the expression of synapse-specific genes.

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CLONING OF THE GENE CODING FOR Mr 60'000 SECRETORY PROTEIN INDUCED BY ANTIMICROTUBULE AGENTS IN *Neurospora crassa*.

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After treatment with antimicrotubule agents, *N. crassa* secretes an Mr 60'000 glycoprotein. This induced glycoprotein was not secreted by untreated cells and was not detected intracellularly by polyclonal antibodies raised against the extracellular form. The gene coding for the protein moiety of the glycoprotein is apparently not transcribed in normal, untreated cells, as shown by immunoprecipitation of the in vitro translation products. We have contructed a cDNA bank using total mRNAs extracted from *N. crassa* cells treated with 1.7 µM benomyl. The Uni-Zap vector containing the β-galactosidase gene (Stratagene) was used for insertion of the cDNA bank and for expression of proteins. We obtained 175 positive clones in 280.000 plaques after screening with polyclonal antibodies raised against the Mr 60'000 protein. A few cDNA from positive clones produced a fusion protein of about Mr 60'000. These fusion proteins were inhibitory for *E. coli* growth while proteins deprived of their N-terminal end had no toxic effect.

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IDENTIFICATION OF A 47 KD CALMODULIN-BINDING PROTEIN AS AN ACTIN-BINDING PROTEIN IN *Neurospora crassa*.

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A 47 KD calmodulin-binding protein (CaM-BP) was purified from the soluble protein fraction of *Neurospora crassa* by chromatography on a CM-sepharose column followed by affinity chromatography on CaM-sepharose 4B. After one dimensional gel electrophoresis (SDS-PAGE), the protein was transferred to a nitrocellulose membrane, then the blot was incubated with a calmodulin-biotinylated and revealed by streptoavidin horseradish peroxydase (SIGMA) with diaminobenzidine. Purified actin from *N. crassa* by affinity chromatography on DNAse I -sepharose 4B, was incubated with the 47 KD (CaM-BP) transferred on nitrocellulose membrane and revealed by antibodies against actin (AMERSHAM N 350). The immunoblots show clearly that this 47 KD (CaM-BP) binds specifically the *Neurospora* actin.

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S100 PROTEINS OF THE HUMAN HEART

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S100α is the major representative of the S100 protein family in the human heart. To date 9 members of this protein family have been characterized by cDNA or protein sequencing, but little is known about their functions. Therefore following questions still remain unanswered: Why are these highly homologous proteins expressed in such a tissue specific manner? What are their targets, if any? What is the function of calcium binding? To be able to give some answers we are characterizing S100 proteins and their function in the human heart.

In a first step we purified S100α and other homologous proteins from human heart tissue. S100α was characterized by immunological methods and by sequencing of tryptic peptides by Edman degradation and mass spectrometry, which will reveal any kind of posttranslational modification. Purification of S100α by HPLC procedures indeed indicates that different forms of S100α may exist in the human heart.

To find new members of the S100 protein family we used degenerated oligonucleotides to amplify the first calcium binding loop by PCR. To bypass stage specific expression patterns we chose genomic DNA as a template for PCR. The amplified products were subcloned and sequenced. The obtained sequences represent five members of the S100 protein family: S100α, S100ß, calcyclin, 42a, and a new member of this family. The subcloned fragments are now used to determine which mRNAs are expressed in the heart and to clone the corresponding cDNAs from human cDNA libraries. With expression studies of the different cDNA clones in cultured heart cells we hope to obtain clues to the function of these proteins.

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SERUM T-KININOGEN LEVELS INCREASE DURING AGING

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We have recently reported an induction of T-kininogen mRNA in aging Sprague-Dawley rats. To further define the role, if any, of this Cysteine protease inhibitor on the aging process, we have now measured the level of the respective protein, both in the serum and within the liver cells. We have found that the protein level is indeed augmented during senescence, although the serum levels are not elevated as dramatically as the mRNA levels (2.5-fold, vs. 8-fold for the mRNA). This can be partially explained by intrahepatic accumulation. Furthermore, measurements at the protein level have allowed us to determine the existence of a positive correlation between the time of increase of the protein level in the serum, and the time of death of the animal. On the other hand, dietary restriction, a known age-prolonging treatment, effectively abolishes the overexpression of the protein in 24-month old animals.

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A PROTEIN WITH EIGHT DOMAINS HOMOLOGOUS TO THE SPHINGOLIPID ACTIVATOR PROTEINS IS EXTRACTED BY BUTANOL FROM *DROSOPHILA MELANOGASTER* LARVAE

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Cell movement and cell-cell interactions are of crucial importance throughout the development of *Drosophila melanogaster*. Treatment of larval membranes with n-butanol solubilizes a limited set of proteins and an activity that promotes aggregation of cultured embryo cells. We have cloned a cDNA encoding one of these proteins, which has also been shown to be the major protein secreted by Schneider S3 cells upon treatment with 20-hydroxyecdysone (Wayne Rickoll, personal communication). Abolishment of zygotic transcription of the cloned gene in a deletion mutant generated from a P-element insertion causes death during embryo development. The deduced amino acid sequence of the cloned protein can be divided into eight homologous domains, each showing homology with the sphingolipid activator proteins and a small family of related lipid-binding proteins. Two forms of the protein can be distinguished immunologically: a 115 kDa form is found in embryos (probably contributed maternally) and in S3 cells, while a 120 kDa form is found in larvae. We are investigating whether alternative mRNA splicing can account for these differences.

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RAT PLASMA SGP-2 IS PARTIALLY ASSOCIATED WITH LIPOPROTEINS

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We have recently identified and characterised a human HDL-associated apolipoprotein (apo), NA1/2. It has since been shown to correspond to simultaeneously identified human proteins (SP-40,40 or cytolysis inhibitor) and bears strong homology to rat sulphated glycoprotein-2 (SGP-2). Using polyclonal antibodies against α and β chains of human NA1/2 and monoclonal antibodies against the β chain, we have identified cross-reacting peptides in rat plasma. Western blots of single dimension SDSPAGE profiles showed bands with Mr of 33kD and 45kD under reducing conditions, and a single band (Mr approx. 80kD) under non-reducing conditions. Similar results were obtained by two dimensional SDSPAGE analyses. Structural data further support our contention that the cross-reacting peptides correspond to SGP-2. Immunoaffinity chromatography using antibody columns against rat apolipoproteins A-I, E and K showed plasma SGP-2 to be partially associated with complexes corresponding to lipoprotein particles when examined by electronmicroscopy. Thus rat SGP-2 parallels its human counterpart in having a plasma component which circulates in association with lipoprotein complexes.

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LIPOPROTEINS AND APOLIPOPROTEINS PRESENT IN HUMAN CEREBROSPINAL FLUID

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Plasma apolipoproteins (apo) are important determinants of blood lipid metabolism. Certain plasma apos are also present in the cerebrospinal fluid (CSF). Their functions are unknown, but it is postulated that they have important roles in lipid homeostasis in the CNS. Thus, they may be useful markers of neurological disorders where cell death releases large quantities of membrane lipids. In the present study, we have characterised lipoproteins and apos from human CSF using (a) immunoaffinity chromatography on apo A-I and apo E antibody columns, (b) electronmicroscopy, (c) 2 dimensional SDSPAGE and Western blotting. Three lipoprotein populations have been identified. Lp (E), retained by the anti-apo E column, contained apo E (100%) and apos A-I, A-IV, D and NA1/2 at 17.4%, 36.6%, 32.7%, 12.5% respectively of their CSF concentrations. Lp (A) was enriched in apo A-I and also contained apos E (77.8%), A-IV (34.0%), D (65.9%) NA1/2 (61.9%). A third, previously unidentified lipoprotein species was also detected; it was particularly rich in apo A-IV (62.6%). These studies clearly demonstrate the compositional heterogeneity, presumably linked to metabolic heterogeneity, of lipoprotein complexes in the CSF.

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MODULATION OF THE STABILITY AND STRUCTURAL DYNAMICS OF F-ACTIN

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The predominant intersubunit contact of the double-helical F-actin filament is made *along* the two long-pitch helical strands of actin subunits, while the spatial extent and mass density of the intersubunit contact *between* the two strands is more tenuous allowing for variable amounts of "lateral slipping" of the two strands relative to each other. We have started investigating factors possibly modulating the degree of lateral slipping and hence the filament stability and structural dynamics. First, we explored the possible effects of inorganic phosphate, P_i, and of the state of hydrolysis of the bound nucleotide on the filament structure and stability by electron microscopy and by determining critical concentrations for polymerization into filaments. Accordingly, the most stable filaments were observed upon replenishing ADP-F-actin with excess fresh ATP, or upon polymerization of (AMP-PNP)-G-actin. ADP-F-actin filaments appeared less stable, and this instability became even more pronounced with ADP-P_i-F-actin. Currently we are exploring conditions that may primarily affect the strength of the long-pitch helix contact. To this end, we have found that filaments polymerized with Ca^{2+} are shorter and appear much more fragile than those polymerized with K^+ and/or Mg^{2+}, suggesting the strength of the long-pitch helix contact to be modulated by the cation bound to the filament.

These observations indicate that actin filaments may play a more active role in acto-myosin-based force generation than hitherto assumed.

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TRANSGLUTAMINASE MEDIATED CROSSLINKING; A NOVEL MECHANISM OF EXTRACELLULAR MATRIX STABILIZATION?

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Laminin/nidogen-complex, a major component of basement membranes, incorporates ^{3}H-putrescine and monodansylcadaverine in presence of guinea pig liver transglutaminase. Label was detected in nidogen in the isolated as well as in the complexed form, but not in laminin. The incorporation reaction proceeds in a time dependent manner at a rate similar to the one achieved with N,N-dimethylcasein, a well known transglutaminase substrate. Saturation of incorporation site(s), as well as comparison with the incorporation in reference proteins, indicated the presence of one high affinity amine-acceptor site in nidogen. Electron microscopy of the reaction products showed that laminin/nidogen-complexes become stabilized in the head to head arrangement, characteristic of the Ca^{2+}induced selfaggregation. Indirect immunofluorescence and detection of transglutaminase activity on unfixed cryosections, revealed an extracellular distribution of the enzyme mainly in collagen rich connective tissue. Co-distribution with laminin/nidogen-complex was not complete but in many locations. Thus, it is likely that transglutaminase mediated crosslinking of laminin/nidogen-complex occurs also *in vivo*.

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THE HETEROPOLYMERIC STRUCTURE OF THE LECTIN, JACALIN.

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Jacalin (from Artocarpus integrifolia seeds), because of its interaction with the D-gal β 1-3 D-galNac moieties present on the hinge region of human IgA1, has been the subject of numerous reports. This lectin exhibits an apparent MW of about 54 kDa and is composed of at least five subunits : α (15 kDa), α' (12 kDa) and β1, β2, β3 (2 kDa each). Some controversy exists as to the number of subunits, and also to the homology of the larger polypeptide chains within the polymer.
Our results show, in contradiction to previous reports, that both α and α' peptides are glycosylated and that upon endo-F digestion each decreases by an apparent MW of 500 daltons. Peptide mapping of the two chains indicates a difference in the primary structure. Extensive sequencing has indicated that, though very similar, both α and α' exhibit a high degree of polymorphism.

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REGULATION OF LAMINFILAMENT DISASSEMBLY BY CDC2-KINASE

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The nuclear lamina is an intermediate filament-type network, which is disassembled when cells enter mitosis. We have shown previously that cdc2-kinase, a major cell cycle regulator, is able to induce lamina depolymerisation when incubated with isolated nuclei. To study the effect of lamin phosphorylation by cdc2-kinase in more detail, lamin filaments were reconstituted from bacterially expressed chicken lamin B_2 protein. We show that cdc2-kinase is able to disassemble these filaments via phosphorylation of two sites that are specifically phosphorylated also during M-phase in vivo; these sites are located in regions flanking the central α-helical rod domain of lamin proteins. Subsequent dephosphorylation of these sites by purified phosphatase 1 (provided by B.Hemmings; FMI) allows reformation of filaments, suggesting that the assembly state of nuclear lamins is mainly controlled by phosphorylation. Interestingly, filaments formed of lamin proteins carrying a mutation in the amino terminal phosphoacceptor site (Ser16->Ala) were resistent to disassembly by cdc2-kinase. No such effects were seen with lamin proteins carrying a corresponding mutation of the C-terminal phosphoacceptor site. These results demonstrate that in vitro the assembly state of lamin B_2 is controlled by phosphorylation of serine 16 in the amino terminal end domain.

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THREE DIMENSIONAL ELECTROPHORESIS OF HUMAN PROTEINS AND THEIR IDENTIFICATION BY MICRO-SEQUENCING.

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The high resolving power of 2D gel electrophoresis has long been recognized. Since the introduction of membranes, both suitable for electroblotting and stable to the Edman degradation, it has been possible to partially sequence proteins directly blotted from gels. If the protein of interest is not abundantly expressed, insufficient material is obtained from a single 2D electrophoresis to yield reliable sequence results. To counteract this problem, the spots from several separations are pooled, the protein is electro-eluted and blotted from a final gel. These manipulations lead to enormous losses and are time consuming. In our laboratories, we have introduced an initial preparative isoelectrofocusing step which allows us to successfully obtain, from a single 2D gel, sequence data on weakly expressed proteins.

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MALATE SYNTHASE FROM GERMINATING SOYBEAN SEEDS: PURIFICATION OF mRNA BY IMMUNOADSORPTION OF POLYSOMES

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Malate synthase (EC 4.1.3.2) is a specific enzyme of the glyoxylate cycle, which commonly occurs in germinating oilseeds. During microbody biogenesis the enzyme is first synthesized on free polysomes and then imported into the glyoxysomes. With a view to determine the glyoxysomal targeting signal of malate synthase we decided to construct a cDNA library enriched in malate synthase sequences. mRNA was prepared from immunoadsorbed polysomes using highly specific polyclonal antibodies. The integrity of polysomal mRNA was controlled by the analysis of the total and immunoprecipited products of *in vitro* protein synthesis. It was estimated that 0.3 % of total polysomal mRNA consists of malate synthase mRNA. In order to amplify the sequences of malate synthase obtained from immunoadsorbed mRNA, cDNA synthesis was associated with polymerase chain reaction amplification.

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SUPPRESSOR tRNA MEDIATED INCORPORATION OF CHEMICAL PROBES INTO PROTEINS

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To probe the molecular environment of a selected site within a protein or polypeptide, a novel crosslinking approach is being developed. Following a published procedure (Bruce, A. G., Atkins, J. F., Wills, N., Uhlenbeck, O. and Gesteland, R. F. (1982) Proc. Natl. Acad. Sci. USA *79*, 7127) , an amber suppressor tRNA was constructed. A method has been developed for the chemical aminoacylation (charging) of this suppressor tRNA with cysteine. The high reactivity of the sulfhydryl group allows subsequent selective modifications of the cysteinyl side chain. Addition of such tRNA to in vitro translation systems (E. coli or wheat germ) leads to incorporation of the derivatized cysteine into proteins in response to an UAG-(amber) codon. Alkylation with a reagent containing a cleavable, photoactivatable function and an isotope of very high specific radioactivity (e.g., 125-iodine) will lead to modified proteins which make unique probes for studying interactions with other macromolecules as well as for identifying nearest neighbors.

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X-RAY STUDY ON THE N-TERMINAL DOMAIN OF THE PHOSPHORYLATING SUBUNIT OF MANNOSE PERMEASE FROM *E. coli*

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The mannose permease complex of *Escherichia coli* consists of three types of subunits (II-P^{Man}, II-M^{Man} and III^{Man}) and mediates the transport of mannose through the cytoplasmic membrane coupled with its phosphorylation. The dimeric III^{Man} is found both in a soluble form in the cytoplasm and complexed with the transmembrane II^{Man} subunits. III^{Man} monomer consists of two structurally unrelated domains: the N-terminal P13 (13 KDa) and the C-terminal P20 (20 kDa). The two domains become transiently phosphorylated at His10 and His175, respectively. They are connected by a flexible hinge and can be separated without loss of stability and catalytic activity, both *in vivo* and *in vitro*. P13 forms an α_2 dimer of which crystals were obtained that belong to the monoclinic space group $P2_1$, with two dimers per asymmetric unit. A full X-ray data set of the native protein has been collected to 2.3 Å resolution using a FAST area detector. Several putative heavy atom derivatives are currently under study.

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X-RAY CRYSTALLOGRAPHIC STUDIES OF VITAMINE B_6-DEPENDENT AMINOTRANSFERASES.

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In addition to the extensive studies of 3D-structure and function of aspartate aminotransferase (AAT) in this laboratory, X-ray studies of the following enzymes are underway: phosphoserine aminotransferase (PSAT) from *Escherichia coli*, γ-aminobutyrate aminotransferase from pig liver and dialkylamino acid decarboxylase from *Pseudomonas cepacia* , a decarboxylating aminotransferase. The crystallographic study of the last two enzymes is still in an early stage, although well diffracting crystals have been grown and characterized. The structure of PSAT, an α_2-dimer, containing 362 amino acid residues and one molecule of the cofactor pyridoxal phosphate (vitamin B_6 aldehyde) per subunit is close to its solution. The course of the polypeptide chain is known for about 90% of the molecule and reveals locally strong structural similarity with AAT, but also distinct differences. About 50% of the amino acid side chains of the molecule have been localized.

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ANALYSIS OF A LAMIN A PROCESSING PROTEASE

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The nuclear lamina is composed primarily of proteins termed lamins. These form a network of intermediate filament-like fibrils that lie subjacent to the inner nuclear membrane. All lamins are synthesized as precursors with a C-terminal CXXM motif similar to the CAAX-box of ras-proteins. At this C-terminus a series of postranslational modifications takes place which enables these proteins to interact with membranes. Specifically, lamins are isoprenylated at the cysteine, after which the three C-terminal amino acids are removed and the cysteine is carboxylmethylated. For A-type lamins these modifications are followed by proteolytic removal of the entire modified C-terminus (about 20 amino acids). This proteolytic event most likely occurs after translocation of the precursor to the nuclear envelope. We are currently analyzing the characteristics of the lamin A-specific protease by exploring its sensitivity to different inhibitors and, by site-directed mutagenesis, we are investigating the sequence requirements for efficient cleavage.

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PREPARATION OF POLYMER-DRUG CONJUGATES USING A SOLID-PHASE SYNTHESIS METHOD

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A simple and rapid method for preparing poly(α-amino acid)-drug conjugates such as thiol-terminated poly-L-glutamic acid-daunomycin macromolecules using a solid phase synthesis method is described. The procedure consists of first preparing an aminoethyl disulfide functionalized glass support. Then a γ-ester-L-glutamate N-carboxy-anhydride monomer is polymerized onto the prepared solid phase. Next, the γ-carboxyl group is deprotected and daunomycin grafted onto deprotected sites. Finally, the disulfide bond between the support and the polymer is thiolyzed by reacting it with mercaptoethanol to produce the desired end product, the thiol-terminated polymer-daunomycin conjugate. Once dialyzed, the conjugate is ready for coupling with a N-maleimido activated protein.

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EVIDENCE FOR OXIDATIVE DETOXICATION OF PTEROSTILBENE AND RESVERATROL BY A STILBENE OXIDASE, LACCASE-LIKE ENZYME SECRETED BY *Botrytis cinerea* Pers.

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On a pectin containing medium, *Botrytis cinerea* produces a hydroxystilbene-degrading enzyme, stilbene oxidase, identified as a laccase. Stilbene oxidase oxidises both pterostilbene and resveratrol, probably by several steps: a hydroxylation followed by an oxidation into several different products. One of them was partially identified as a dimer of pterostilbene. This oxidation detoxifies the hydroxystilbenes. Stilbene oxidase was purified on DEAE-Sepharose FF and then on a laccase affinity gel, syringyl-EAH-Sepharose 4B. Six protein bands from the affinity gel active fraction were separated on IEF-PAGE (pH 3-10). Their pI were comprised between 3.5 to 4.7. The relative molecular weights were determined by SDS-PAGE (10%). Three major bands with KD 97.6, 86.2 and 64.5 were revealed by silver staining method.

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SIGNAL FOR ADDITION OF A PHOSPHOLIPID MEMBRANE ANCHOR ON THY-1 GLYCOPROTEIN.

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The Thy-1 glycoprotein, a major surface component of rodent thymocytes and neurons, is anchored in the cell membrane via a phosphatidyl-inositol containing glycolipid (GPI). A common feature of GPI-anchored proteins is a C-terminal hydrophobic stretch of amino-acids that can be deduced from the cDNA sequence but is absent on the mature protein. The signal that directs cleavage of this C-terminal segment and addition of the GPI anchor is not known but recent studies have focused on the identification of the primary structure and the conformation of this critical region. We have performed deletions and substitutions of amino-acids in the C-terminus region of Thy-1 and transfected these mutated genes into Hela cells under the control of SV 40 early gene promoter. Stable clones will be caracterized with respect to cell surface expression, GPI anchoring and processing of the Thy-1 protein. Expression studies on these various constructs should help defining the structural signal required for the anchorage of membrane proteins via a glycolipid.

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LOCALIZATION OF α-ACTININ AND VINCULIN IN ADHERENT HUMAN NEUTROPHILS

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We have demonstrated the presence of the putative actin-membrane linker protein vinculin and the actin-crosslinking protein α-actinin in human neutrophils (Eur. J. Cell Biol. 49, 366, 1989). We have now studied the location of these proteins in human neutrophils adhering to glass, using immunofluorescence. F-actin was visualized by rhodamine-conjugated phalloidin. Vinculin was concentrated in small patches at the cell periphery, partially colocalizing with cellular areas appearing black in interference reflexion microscopy (IRM). These black areas may correspond to areas of strong adhesion of the cell with the substrate. In these areas, vinculin colocalized with F-actin. α-Actinin colocalized with F-actin in retraction fibers, which also correspond to dark IRM sites. Both proteins may thus be involved in actin-membrane linkage in neutrophil adhesion sites. In some cells, F-actin rich cell protrusions were also enriched in α-actinin. Cytochalasin D, which disturbs the actin network, displaced vinculin from the patches and induced cocapping of α-actinin and F-actin.

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SMALL AMPHIPATHIC POLYPEPTIDES: THE DECISIVE STRUCTURAL ELEMENTS FOR FAR-RED SHIFTED BACTERIOCHLOROPHYLL-B CONTAINING MEMBRANE-BOUND ANTENNA COMPLEXES ?

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In addition to the well-characterized α- and β-antenna polypeptides of bacterial intra-membrane bound antenna complexes a third type (γ-apoprotein) of polypeptide has been isolated exclusively from species containing bacteriochlorophyll b and showing in-vivo absorption maxima mainly at around 1020 nm. Three such species have been investigated: *Rhodopseudomonas viridis, Ectothiorhodospira halochloris* and *E. abdelmalekii.* The γ-apoprotein of *Rp. viridis* is 36 amino acids long and those from the other two species 29 a.a.residues. For all three species there is good evidence that there are multiple forms (2 to 4 γ-apoproteins with different sequences) present. It is postulated that these amphipathic peptides are involved in i) forming regular arrays of light-harvesting systems as observed in BChl b containing bacteria (structural role) and ii) shifting the absorption of BChl b (in vitro at ~ 790 nm) up to 1020 nm (functional role).

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Pregnancy-Associated Plasma Protein B (PAPP-B) and its Biochemical Properties.

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PAPP-B was first described by **T.M.Lin** *et al.* (Archs. Allergy Appl. Immunol., 1978, *57*, 294-303). Combining LPLC with HPLC, we were able to identify a single protein with antigenical reactivity on western blot against anti PAPP-B antibodies. The molecule was characterized as a glycoprotein with a MW of 1,300 kD, composed by three subunits each built up of 6 elementary units of 74 kD and a carbohydrate content of 12.2%, its pI was measured at 5.3. Anti PAPP-B antibody specifically precipitated radioactive proteins *de novo* synthesized by placentae perfused with ^{35}S-Met. The same antibody produced a positive reaction in trophoblastic sections stained with the avidin-biotin-peroxidase complex. Epidermis of the skin, blood vessels, connective tissue and adipocytes also stained positively, no reactions were observed in hepatocytes. We conclude that PAPP-B is of trophoblastic origin but the ectopic reactions have to be further investigated.

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Clinical Studies on Pregnancy-Associated Plasma Protein B (PAPP-B).

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PAPP-B was biochemically described by **T.M.Lin** *et al.* (Inter. Archs. Allergy Appl. Immunol., 1978, *57*, 294-303). Their clinical studies indicated that concentration of PAPP-B in maternal serum increases in pregnancy and decays after delivery with a half life shorter than 24 hours.

Our first attempts to prepare a test (conjoint ELISA) able to measure PAPP-B in biological fluids indicated an increasing concentration of the protein in maternal serum during the pregnancy with a peak at the 35th pregnancy week. No reaction was detected between anti human PAPP-B and sera of pregnant mice, cats, dogs and pigs. The half life post partum of PAPP-B seemed to be longer than proposed by Lin (ca. 48 h). The compartimentation of PAPP-B indicated a similar concentration in maternal serum, retroplacental serum and in fetal cord serum. Very low PAPP-B quantities were found in urine and in amniotic fluid. Normal male and female sera contained lower PAPP-B levels than normal pregnancy sera.

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THE ECTODOMAIN OF SFV SPIKE PROTEINS IS REQUIRED FOR PROTON TRANSLOCATION

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Exposure of Semliki Forest virus (SFV)-infected Aedes albopictus cells to mildly acidic pH (< 6.2) results in a proton influx into the cell which correlates with cell-cell fusion. This proton influx is most probably mediated by the viral spike proteins which are expressed in the plasma membrane. That viral spike proteins can function as proton channels was demonstrated on intact virions using the nucleocapsid as a pH indicator. Nucleocapsids exposed to low pH undergo an irreversible conformational change which results in a shrinking and an altered sedimentation coefficient as shown by electron microscopy and sedimentation analysis, respectively. In contrast to intact virions proteolytically digested SFV particles contained nucleocapsids with identical size and sedimentation coefficient after low- and neutral pH treatment. Analysis of the membrane associated protein fragments of shaved particles revealed that proteolysis is complete and leaves only the membrane anchors of the spike proteins.

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LIMITED TRYPTIC DIGESTION OF TETRAMERIC BOVINE CAUDATE NUCLEUS ACETYLCHOLINESTERASE YIELDS ACTIVE MONOMERS

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Bovine caudate nucleus acetylcholinesterase (AChE) is an amphiphilic enzyme consisting of four catalytic subunits with identical primary sequences and a membrane anchor of scarcely defined structure. Two of the subunits are interconnected via disulfide bonds, the other two are attached via disulfide bonds to the common membrane anchoring domain. Reduction and alkylation of the tetramer in conditions which retain activity does neither result in monomerization of AChE nor in a hydrophilic enzyme. In contrast, incubation of AChE with trypsin in the presence of edrophonium chloride renders the enzyme hydrophilic and leads to monomerization. SDS-PAGE of this preparation in non-reducing conditions revealed a decrease in the subunit molecular mass of around 2 kDa. N-terminal sequencing of the enzyme before and after trypsin treatment yielded identical N-terminl showing that the enzyme was monomerized subsequent to C-terminal tryptic cleavage. Upon autoradiography of ^{125}I-TID labelled enzyme, the anchoring domain can be detected at around 18 kDa showing that the anchor was released from the subunits. From our results we conclude that the last Cys within the primary sequence is involved in intersubunit disulfide bonding as well as in the attachment of AChE to the membrane anchor.

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PURIFICATION AND CHARACTERIZATION OF THE RECOMBINANT 37KDA SOLUBLE CD23

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The lymphocyte low-affinity receptor for IgE (Fc_ϵRII) CD23 is involved in 1) promotion of B-cell growth (BCGF), 2) isotype-specific regulation of IgE synthesis. CD23 is a 45kDa monomeric glycosylated protein with an inverted membrane orientation. The IgE binding site of CD23 is located in the animal lectin homology domain in the C-term end of the molecule. Due either to autoproteolysis or to membrane associated proteases, the receptor is cleaved into soluble fragments (sCD23). The 37kDa fragment is a short-lived intermediate which is rapidly degraded to the more stable 25kDa molecule. Most of the reported studies have been performed on culture supernatants or affinity purified material of the EBV-transformed lymphoblastoid cell line RPMI 8866 containing traces of the various sCD23. We have obtained highly purified recombinant 37kDa sCD23 using a baculovirus/insect cell expression system. The physico-chemical characterization of this molecule and the IgE binding activity will be presented.

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CHARACTERISATION OF RECOMBINANT HUMAN INTERLEUKIN-5 PRODUCED IN ESCHERICHIA COLI

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The gene coding for human interleukin-5 was synthesized and expressed in Escherichia coli under control of a heat-inducible promoter. High-level expression, 10-15% of total cellular protein, was achieved in E. coli. The protein was produced in an insoluble state. A simple extraction, renaturation and purification scheme is described. The recombinant protein was found to be a homodimer, similar to the natural murine-derived protein. Each subunit contains two cysteine residues that form two inter-subunit disulphide bonds. The topology of the disulphides in recombinant human interleukin-5 produced in E. coli was studied by proteolytic digestion and peptide mapping. Disulphide linked peptides containing cysteine 42 linked to cysteine 84 were isolated. This indicated that cysteines 42 and 84 of one subunit were linked in an antiparallel manner to cysteines 84 and 42 of the other subunit.

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ELONGATION ARREST DOMAIN OF THE SIGNAL RECOGNITION PARTICLE.

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The Alu-domain of the signal recognition particle (SRP) contains an elongation arrest function which effects a specific but transient inhibition of the synthesis of presecretory proteins. It consists of two proteins, SRP9 and SRP14, and the Alu-sequences of SRP RNA. cDNA clones of SRP9 and SRP14 have been isolated. Both proteins are highly charged and have no homology to other RNA-binding proteins. SRP9 and SRP14, derived in vitro from their cDNA clones, restore elongation arrest activity to a partially depleted SRP, SRP-9/14, which is deficient in this function.The cDNA clones have been used for studying the RNA binding characteristics of the proteins. We found that the presence of both proteins was required to form a stable RNA-protein complex. Thus, the presence of both proteins is essential for the assembly of a functional domain. Using several different approaches, we have examined the structural components of SRP RNA that are involved in binding SRP9/14.We found that all essential sequence elements are contained within the Alu-portion of SRP RNA. Specifically three sequence blocks are in direct contact with SRP9/14. One of them is conserved in primary sequence throughout evolution from bacteria to archebacteria and higher eucaryotes.

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EXPRESSION OF ACTIVE CHICKEN MITOCHONDRIAL CREATINE KINASE IN *ESCHERICHIA COLI*

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We have expressed a cDNA clone of the chicken mitochondrial creatine kinase (Mi_b-CK) in *E.coli* from an expression vector carrying an inducible T7 RNA polymerase promoter (as described by Studier and co-workers). After 3-4 hours of induction the Mi_b-CK constituted roughly one third of the total cellular protein. The Mi_b-CK was expressed in a soluble, octameric form and showed high specific activity. The wild type form of the protein is being studied biochemically. Mutant alleles of Mi_b-CK are currently constructed and expressed as well. Targets for site directed mutagenesis are the residues which where identified by reactive substrate analogues as important for the enzymatic activity of the enzyme. An *in situ* color reaction assay is developed in order to identify mutant alleles expressing inactive forms of the Mi_b-CK in *E.coli* after random mutagenic procedures.

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A LOW MOLECULAR WEIGHT CYTOSOLIC COMPONENT OF HUMAN NEUTROPHIL NADPH OXIDASE TRANSLOCATES TO THE PLASMA MEMBRANE DEPENDING ON THE STIMULUS

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To further understand the assembly of an active NADPH oxidase complex that requires molecules from the membrane and the cytosol, a polyclonal antibody was raised against the pI 5.0 fraction from solubilized active enzyme resolved by preparative isoelectric focusing electrophoresis. Immunoanalysis of the NADPH oxidase from active and resting cells revealed the presence of a 12-14 kDa protein mainly detected in the cytosol of resting cells, but not in the cytosol of activated cells. In contrast, this protein appeared in the membrane of activated but not resting cells, suggesting its translocation upon cellular activation. This translocation was found to depend on the nature of the stimulus and maximally seen with opsonized zymosan. To prove that the cytosolic protein was translocated, sequence analysis of the 25 N-terminal amino acids of the cytosolic and membrane-bound proteins was undertaken and found to have an almost complete identity. In conclusion, a new cytosolic component has been identified that could play an important role in NADPH oxidase activation.

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7-SUBSTITUTED PTERINS IN MAMMALS

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Recently a new form of atypical phenylketonuria has been discovered. It is characteristic for this form that these patients excrete 7-substituted pterins: 7-iso-biopterin (primapterin), 6-oxo-7-iso-biopterin and 7-iso-neopterin (anapterin). The 7-substituted pterins were so far only known to occur in insects.
We postulated an enzyme defect in the hydroxylation of phenylalanine to tyrosine, a reaction where tetrahydrobiopterin (BH_4) acts as a cofactor. In the hydroxylation cycle two other enzymes are involved: a dehydratase and a reductase. During the hydroxylation BH_4 reacts to 4a-peroxy-BH_4, then to 4a-carbinolamine. In the presence of dehydratase the quinonoid-BH_2 is formed and is then reduced back to BH_4 by the dihydropteridine reductase. Under our assay conditions (alkaline pH, absence of dehydratase) we observed formation of 7-substituted pterins *in vitro*. This fact can be explained by an accumulation of 4a-carbinolamine, and a ring-opening to form a spiro-compound. The later can be cleaved in two ways forming either 6- or 7-substituted pterins.
Our results suggest a dehydratase defect in these patients. We plan to measure dehydratase activity in the small intestine biopsy of the patient and of the controls. For this purpose we develop a sensitive dehydratase assay.

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SITE-DIRECTED MUTAGENESIS ON SPINACH CHLOROPLAST THIOREDOXIN f

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Thioredoxins are important regulatory proteins in photosynthetic systems. Thioredoxin f is the specific activator protein of chloroplast fructose-1,6-bisphosphatase. In order to dispose of sufficient amounts of this protein, which is rather difficult to purify from spinach leaves, to study structure-function relationship thioredoxin f has been overexpressed in E.coli. The protein, either isolated from leaves or from the bacterial cells, is rather hydrophobic and difficult to obtain in concentrated solution. We are therefore trying to modify its primary structure by site-directed mutagenesis 1) to render the protein more soluble and 2) to find which residue(s) are responsible for the high target specificity of thioredoxin f.

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Characterisation of the enzymes catalysing the degradation of toluene to benzoic acid in Pseudomonas putida pWW0

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The TOL plasmid pWW0 of Pseudomonas putida encodes the enzymes catalysing the degradation of alkylaromatic compounds to Krebs cycle intermediates. The first three enzymes of this catabolic pathway; namely xylene monooxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase; which catalyse the oxidation of toluene to benzoic acid, were characterised.

Xylene monooxygenase was determined to consist of two different subunits coded for by xylA and xylM. The xylA gene product was purified and characterised. It contains one equivalent of tightly bound FAD and an [2Fe-2S] cluster.

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are both NAD^+-dependent dehydrogenases specific for aromatic substrates. The kinetic parameters, substrate specificities, and other characteristics of the two dehydrogenases have been determined.

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A MICROVILLUS MEMBRANE METALLO-ENDOPEPTIDASE (PPH) FROM HUMAN SMALL INTESTINE: MOLECULAR AND BIOCHEMICAL PROPERTIES

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Paba peptide hydrolase (PPH) is used to describe a microvillar enzyme activity responsible for non-pancreatic cleavage of N-Bz-L-Tyr-p-amino benzoic acid (Bentiromide, PFT test). Biochemically, PPH is clearly similar to mouse-MEPRIN and rat endopeptidase-2, two metallo-endopeptidases found in kidney microvillus membranes.
PPH has been immuno-purified and analysed by SDS-PAGE. A double band was obtained under reducing conditions (Mr~100 kDa). The two species (termed PPH1 and PPH2) have CNBr-peptide maps with distinct differences, and show non-identical N-terminal amino acid-sequences. Oligonucleotide probes corresponding to these sequences have been used to screen a cDNA library from human small intestine.
One partial cDNA clone encoding PPH2 has been isolated and characterised.
Work is in progress to elucidate the complete primary structure of PPH and to study the relationship with the kidney peptidases found in rodents.
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PURIFICATION AND CHARACTERIZATION OF CHORISMATE SYNTHASE AND CLONING OF ITS cDNA FROM *CORYDALIS SEMPERVIRENS*

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Chorismate synthase (EC 4.6.1.4) catalyzes the last step in the shikimate pathway, which is common to the biosynthesis of all three aromatic amino acids. The enzyme, purified from a cell suspension culture of *Corydalis sempervirens*, has been characterized, and the amino acid sequence of two tryptic peptides has been determined. An antiserum directed against the purified enzyme was used to screen a *C. sempervirens* cDNA expression library. One positive clone was identified and then used to rescreen the library which allowed the isolation of several full length clones. The N-terminal region of the deduced amino acid sequence shows similarities to known plastidic transit peptides. The homology of the mature enzyme to known bacterial sequences is about 50%.

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IMMUNOCHEMICAL CHARACTERIZATION OF NAD(P)H:QUINONE REDUCTASE AND CARBONYL REDUCTASE

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Quinones and structurally related compounds exert toxic effects in biological systems. These compounds are reduced by single-electron transferring enzymes to unstable semiquinones which subsequently react with oxygen generating superoxide anions and other cytotoxic oxygen species. Alternatively, quinones can undergo two-electron reduction to hydroquinones which are conjugated and excreted. Enzymes mediating the two-electron transfer may thus protect the cell against quinone-induced toxicity. Two enzymes, carbonyl reductase (CR, EC 1.1.1.184) and NAD(P)H:quinone reductase (NQR, EC 1.6.99.2) promote the two-electron reduction in animal tissues. Both enzymes catalyze the reduction of virtually the same quinone substrates, but are structurally not related. Using immunochemical techniques, we were able to discriminate the two reductases. Antibodies against CR and NQR were raised in rabbits and their specificity was tested using an ELISA, immunodiffusion and western blots. No cross-reactivity was detectable. For quantitative determinations an indirect competitive ELISA was developed. Incorporation of organomercurials into the assay system increased the sensitivity.

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Towards the expression of a mammalian prolylendopeptidase in yeast.

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Prolylendopeptidase is a 80 kDa serineprotease, which cleaves polypeptidchains carboxy-terminal to proline residues. The aim of our studies is to investigate in this unique reaction-mechanism and the possible use of prolylendopeptidase for the addition of prolines in enzymatic peptide synthesis. Expression of the enzyme in yeast shall provide us with the required amount of active protein.
A cDNA-clone (P2), containing the entire coding region for prolylendopeptidase was kindly provided by Denise Rennex (Friedrich Miescher-Institut, CH-Basel). P2 was inserted into two different expression vectors: i) pRA23, which contains the GAL1 promoter, the carboxypeptidase Y (CPY) pre- and prosequence separated by a unique Bam H1 restriction site from the CPY transcription termination region. P2 was inserted with the correct reading frame into this Bam H1 site (=> pU). The identity of the sequences in the plasmids was substantiated by dideoxy sequencing and yeast cells (W2579, vpl⁻) were transformed. ii) pMA91, which contains the PKG promoter separated by a unique Bgl II site from the PGK transcription termination region. This site was used to insert P2 alone (=> pI) and P2 attached to the invertase(=> pN) or phosphatase (=> pH) signal sequence. DBY 746 yeast cells were transformed with the correct plasmids.
While the plasmids (pU, pH, pN) are designed to secrete recombinant prolylendopeptidase into the culture medium, the construction (pI) should result in the intracellular expression of the protease. The last construction will prevent folding and glycosylation problems, which might occur, when a protein travels through the secretory pathway of the yeast. The amount and activity of recombinant prolylendopeptidase is presently analyzed with synthetic dipeptides (Z-Gly-Pro-pNA and Z-Gly-Pro-βNA).

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ENZYME CHARACTERIZATION OF PBMN CELLS IN DRUG ABUSERS WITH AND WITHOUT HIV SEROCONVERSION

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Changes induced by drug adduction on plasma membrane enzymes involved in the reception and transmission of antigenic stimuli and various hormones were investigated in peripheral blood mononuclear cells (PBMN) of non-infected and HIV-infected drug abusers . PtdInositol Phospholipase-C of HIV-infected cells showed a more acidic pHo but a lower activity than control PBMN and HIV-negative cells. The lowest 5'-Nucleotidase activity was measured in the HIV-positive cells whilst HIV-negative cells show an activity somewhat higher but lower than in normal PBMN. The activity of neutral endopeptidase (NEP; enkephalinase E.C.3.4.24.11), active on opioids, neuropeptides, substance P, interleukin-1, and with an aminoacid sequence identical to CALLA antigen, was found however, absent or very low in PBMN cells of HIV+ drug abusers who showed CALLA positivity on the cell surface (ranging from 3 to 29 %) detected by an anti-CD10 Mab. NEP activity was induced in previously PHA stimulated PBMN with cocaine but not with morfine after 4 days incubation, both compounds used at a concentration (0.3 μg/10^6 cells) close to that found in plasma of drug abusers.

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CONFORMATION IN AQUEOUS SOLUTION OF PENTAPROLINE CONTAINING PEPTIDE THAT BINDS TO MHC CLASS I MOLECULES.

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T lymphocytes expressing variable cell surface antigen receptors recognize "processed" forms of antigen, presented on the surface of antigen presenting cells in association with molecules of the major histocompatibility complex (MHC). Naturally processed antigenic peptides can be replaced by synthetic ones. Truncation or amino-acid substitution of the antigenic peptide HLA A24 170-182 (RYLENGKETLQRA) that is recognized by A24 specific T-cells in association with the H-2K^d (K^d) class I MHC molecule suggests that the three residues Y-171, T-178 , L-179 allow the peptide to interact with the K^d restriction element. The analog AYPPPPPTLA is an active competitor . The pentaproline was designed to play the role of a rigid spacer. Alanine residues were added to avoid a charged N and C termini on the Y and L residues. Nuclear magnetic resonance methods (NMR) allow us to demonstrate that in aqueous solution the Y2-P3 bond gives rise to a trans-cis isomerization (67% for trans, 33% for cis). When this this bond is in trans, the pentaproline spacer assumes an all trans conformation, when it is in cis the next P3-P4 bond shows a trans-cis isomerization in a ratio that cannot be precisely quantified. However, in aqueous solution the pentaproline spacer adopts an all trans conformation in more than 80% of the molecules.

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DEFINED OLIGOMERS FOR DRUG THERAPY USING AN ANTIBODY-MEDIATED DELIVERY SYSTEM

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A new series of defined synthetic oligomers are described for drug therapy. The oligomers consist of a peptide backbone with pendant drugs attached to side chains through hydrazone bonds. The linkage of the drug-bearing oligomer to antibody is made through a functional group attached to the amine terminus of the oligomer. This functional group reacts specifically with a complementary chemical group placed on the antibody (or fragment) by chemical or enzymatic means in a prior step. Site-specific modification is thereby achieved in this two-step approach and use of a defined oligomer leads to a relatively homogeneous product. Release of drug is achieved through spontaneous decomposition of the hydrazone bonds after internalization in the target cell or external hydrolysis within its microenvironment. The preparation and properties of a defined oligomer carrying a pharmacologic agent will be described.

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SITE-SPECIFIC MODIFICATION OF A MONOCLONAL ANTIBODY DIRECTED AGAINST CANCER CELLS

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When monoclonal antibodies are modified for diagnostic and therapeutic purposes, much is to be gained if such modification can be made site-specific, particularly if the site of the modification is far from the antigen binding region. A method based on enzyme assisted reverse proteolysis is described, where a linker group, having a unique chemistry is attached specifically to the carboxy terminus of the heavy and light chains of the antibody. The presence of the linker group allows the specific attachment of a wide range of therapeutic or diagnostic agents possessing a chemistry complementary to that of the linker group. To determine whether the modified antibody is still immunoreactive, *in vitro* binding tests are performed. The biodistribution of the modified antibody is also studied in a nude-mouse xenograft system.

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IN VITRO IMMUNIZATION SYSTEM FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST ANTIGEN WITH HIGHLY CONSERVED STRUCTURES

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For diagnostic uses, we intended to prepare monoclonal antibodies with specificity for the enzymes of the *de novo* biosynthesis of tetrahydrobiopterin cofactor (BH_4), which is essential for the biosynthesis of the neurotransmitters dopamine and serotonin. The BH_4 biosynthetic enzymes are present in all mammals and lower animals, even in bacteria, and their structures seem highly conserved.
To raise a good immune response *in vivo* is also difficult due to the very small amounts of protein being available after purification. For successful immunization we used the following *in vitro* system : Nonimmune splenocytes were incubated with the antigen in the presence of supernatants from MLC (33 %) and EL-4 (25 %, after stimulation of the thymomas with phorbol ester) in a total volume of 10 ml/spleen. As basic medium we used IMDM-GM free of serum and antibiotics. After a 5 day immunization period the cell population was checked for viability and B-blast formation. Fusion was performed with Ag 8.653 in 50 % PEG 4000 in GKN at a spleen : myeloma ratio of 2:1. Cells were plated on peritoneal macrophages on 96-well plates. For GTP cyclohydrolase as antigen (from *E. coli*, 0.1 µg/ml) a total of 3 µg were used in the immunization system. Hybrid growth was observed in 85 % of the wells and 35 positive clones were selected.

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IN VITRO IMMUNIZATION WITH ANTIGEN DIRECTLY BLOTTED FROM SDS-PAGE TO PVDF MEMBRANES

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6-Pyruvoyl tetrahydropterin synthase (PTPS) is one of the enzymes involved in the biosynthesis of tetrahydrobiopterin (BH_4), the cofactor of the aromatic amino acid hydroxylases. PTPS deficiency is the most common form of BH_4-deficiency and is a very heterogeneous disease. For the study of this disease at the gen level we produced monoclonal antibodies for the screening of cDNA libraries as follows : PTPS partially purified from human pituitary glands was blotted from SDS-PAGE to PVDF membranes and stained briefly with Ponceau S. The PTPS bands were then cut from the membranes and sterilized by gamma irradiation.
After adapting the membranes to the *in vitro* medium system they were placed into the culture flask containing IMDM-GM and lymphokine supplements. To enhance the immune response synthetic muramyl dipeptide (MDP) was added to a total concentration of 10 µg.
The spleen cell preparation was then layered onto the membranes and the system incubated for 5 days at 8 % CO_2 and 37 °C. The fusion was carried out in 50 % polyethylene glycol (PEG) 4000 at standard conditions. The first hybridoma growth was observed 4 days after the fusion. 125 clones were screened from the growing clones by ELISA against PTPS. 50 % of the tested clones showed high specificity.

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PREDICTED STRUCTURE AND COVALENT ASSOCIATION OF J CHAIN WITH POLYMERIC IgM.

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J (joining) chain is an acidic 15kD polypeptide covalently linked to the penultimate cysteine residue of polymeric IgA and IgM heavy chains. Its role in the poly-Ig assembly and secretion by plasmocytes as well as in the binding to poly-Ig receptors on epithelial cells still remains controversial. Furthermore, to date, the assignment of intra-cystines within J chain as well as of inter-disulfide bridges with polymeric Igs has not been made. Two hypothetical 3-D models have been proposed on the basis of the known primary structure. In order to test the validity of such models and in particular to determine the exact disulfide bond pairings, we have isolated disulfide-bridged fragments derived from a CNBr digest of J chain-containing Waldenström IgM. Micro-sequencing of such peptides revealed disulfide pairings inconsistent with both models, except for the conserved intra S-S bond between positions 91-100. The comparison of our results with the two existing hypothetical models will be discussed.

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NATURALLY OCCURING ANTIBODIES AGAINST IL-1 IN HUMAN PLASMA

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During the development and testing of a radioreceptor assay (RRA) for human IL-1, we have detected and identified the presence of autoantibodies to IL-1 in normal human plasma (NHP). The RRA is based on the competition between human labeled rIL-1α and standard or unknown quantities of IL-1α or IL-1β for binding to a limited amounts of IL-1 receptor (IL-1R) isolated from the EL4 mouse thymoma cell line. 20 out 100 NHP from unselected blood donors were found to completely inhibit the binding of labeled IL-1α to his receptor suggesting the presence of either abnormal amounts of IL-1 or of a plasma factor binding to the labeled IL-1α. Inhibiting plasma samples preincubated with labeled IL-1α and filtered on a Sephadex G200 column showed the presence of labeled complexes eluted with an apparent molecular weight of 150-200kDa. Furthermore these plasma factors could be eliminated by treatment with protein A-Sepharose indicating the presence of IgG antibodies directed against IL-1. Screening of 200 NHP samples by a polyethylene glycol precipitation, which detects complexes between antibodies and labeled IL-1) confirmed that 20% of NHP contain detectable IgG antibodies to IL-1α, while only 2% of NHP contain antibodies to IL-1β. (* Supported by Bühlmann Laboratories AG)