Labeling of the Glycoprotein Subunit of (Na,K)ATPase with Fluorescent Probes†

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ABSTRACT: Sodium plus potassium activated adenosinetriphosphatase [(Na,K)ATPase] is composed of a catalytic subunit (α) and a glycoprotein subunit (β) of unknown function. A method has been developed to label the β subunit of purified dog kidney (Na,K)ATPase with fluorescent probes. The method consists of oxidation of β-subunit oligosaccharides, reaction of the resulting aldehydes with fluorescent hydrazides, and reduction of the hydrazones and unreacted aldehydes with $NaBH_4$. Two oxidation methods were compared. Simultaneous treatment with neuraminidase and galactose oxidase did not inhibit significantly (Na,K)ATPase activity and allowed insertion of up to 11 mol of probe per mol of β. In contrast, oxidation of (Na,K)ATPase oligosaccharides with periodate resulted in 50–80% inhibition of the (Na,K)ATPase activity with low or undetectable labeling. Eleven commercial probes and two novel hydrazides were tested for labeling of (Na,K)ATPase treated with galactose oxidase and neuraminidase. Eight probes did not label (Na,K)ATPase but labeled red cell ghosts oxidized with periodate. Four probes labeled β specifically but either adsorbed to the membrane tightly, or cross-linked the β subunits, or formed unstable adducts. Lucifer yellow CH labeled β specifically without membrane adsorption. Labeling stoichiometries from 1 to 11 mol of lucifer yellow CH per mol of β were obtained without inhibition of (Na,K)ATPase activity and without significant alteration of the anthroylouabain binding capacity or its association and dissociation kinetics. Anthroylouabain specifically bound to the lucifer-labeled (Na,K)ATPase had a decreased quantum yield, probably due to resonance energy transfer. This suggests that the sites of lucifer attachment on β are within energy transfer distance from the cardiac glycoside site on α. The fluorescence emission maximum and anisotropy of the labeled enzyme indicate that the sites of lucifer yellow CH attachment are in an aqueous environment and have a high degree of motional freedom in the nanosecond time scale. This is consistent with the postulated location of lucifer yellow CH near the nonreducing end of the β-subunit oligosaccharides. Possible applications are discussed.

Sodium plus potassium activated adenosinetriphosphatase [(Na,K)ATPase][1] is the enzyme that maintains Na^+ and K^+ gradients across animal cell plasma membranes by coupling ion pumping to the hydrolysis of ATP [see Cantley (1981) and Jørgensen (1982) for recent reviews]. The minimal functional unit of the enzyme is composed of a heterodimer of α and β subunits (Moczydlowski & Fortes, 1981b; Brotherus et al., 1981; Craig, 1982). The α subunit is a transmembrane polypeptide of 93 000–120 000 daltons that contains the cardiac glycoside site and the ATP site on the extracellular and intracellular sides, respectively. The α subunit undergoes ligand-dependent conformational changes and reversible phosphorylation by ATP and inorganic phosphate. For these reasons, α is considered the catalytic subunit. The β subunit is a glycoprotein with a protein mass of 37 000–56 000 daltons; β is close to the cardiac glycoside site of α (Hall & Ruoho, 1980) and appears to vary in sensitivity toward proteolysis depending on the presence of ligands of the enzyme (Lo & Titus, 1978; Koepsell, 1979). The function of β is unknown, although it has been postulated that β directs the insertion of α into the membrane (Sabatini et al., 1981).

A fluorescent label that is specific for β may be useful to study the structure and mechanism of (Na,K)ATPase. The use of fluorescent probes which are specific for α has yielded important insights into the structure and mechanism of the enzyme. In contrast to the variety of chemical modification techniques available for α, very few reagents label β, and none of these are specific for this subunit. One approach to label β would be to modify its oligosaccharide portion. Previously, oxidation techniques directed toward carbohydrates have been used to attach fluorescent probes to rhodopsin (Shaper & Stryer, 1977), the erythrocyte sialoglycoproteins (Abraham & Low, 1980; Cherry et al., 1980), RNA (Reines & Cantor, 1974), and the 50S ribosomal protein LT2 (Zantema et al., 1982).

This paper describes the synthesis of two fluorescent hydrazides and a method to couple hydrazides specifically to β-subunit oligosaccharides of purified dog kidney (Na,K)-ATPase without significant inhibition of (Na,K)ATPase activity, loss of ouabain sites, or subunit cross-linking. The applications and limitations of the various probes and alternate oxidation methods are discussed. Preliminary reports of this

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[1] Abbreviations: (Na,K)ATPase, sodium plus potassium activated adenosinetriphosphatase; AO, anthroylouabain; DCH, dansylcarbohydrazide; DCTAF, 5-[(dicarbohydrazidotriazinyl)amino]fluorescein; DTT, dithiothreitol; FTC, fluoresceinthiosemicarbazide; Hepps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; MBA, 3-methoxybenzyl alcohol; Mops, 3-(*N*-morpholino)propanesulfonic acid; lucifer, lucifer yellow CH, *N*-[(hydrazinocarbonyl)amino]-4-amino-3,6-disulfonato-1,8-naphthalenedicarboximide; PBH, pyrenebutyrylhydrazide; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Pipes, 1,4-piperazinediethanesulfonic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; 1,5-dansic acid, 5-dimethylaminonaphthalene-1-sulfonic acid.

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work have been presented (Lee & Fortes, 1982, 1983).

Materials and Methods

Materials. Catalase, galactose oxidase, lactate dehydrogenase, neuraminidase, pyruvate kinase, and NADH were from Sigma. Bovine serum albumin was from Miles. Phosphocellulose, Biosil A, acrylamide, bis(acrylamide), and SDS were from Bio-Rad. Carbohydrazide, 3-methoxybenzyl alcohol, and lucifer yellow CH were from Aldrich. All buffers were from Calbiochem and were Ultrol grade. DTAF was from Research Organics Inc. Ethidium monosemicarbazide and proflavin monosemicarbazide were provided by Dr. Constance Larkey; all other fluorescent amines and hydrazides were from Molecular Probes and were used without further purification.

Enzyme Purifications and Assays. Crude *Dactylium dendroides* galactose oxidase was separated from protease contaminants by a modification of the procedure of Tressel & Kosman (1980a). All steps were carried out at 0–4 °C; polyethylene columns and test tubes were used to minimize galactose oxidase adsorption. Galactose oxidase (450 units) was dialyzed against 5 mM sodium phosphate, pH 7.8, loaded on a 2 cm × 2.5 cm phosphocellulose column, washed with 5 mM sodium phosphate, pH 7.8, and equilibrated with 20 mM sodium phosphate, pH 7.8. A linear gradient of 20–100 mM sodium phosphate, pH 7.8, eluted the galactose oxidase activity at approximately 35 mM sodium phosphate; bovine serum albumin (1 mg/mL) was added to the column fractions to stabilize the activity. The fractions containing the highest activity were divided into aliquots, frozen in liquid nitrogen, and stored at −70 °C until use. Galactose oxidase activity was assayed by the oxidation of 3-methoxybenzyl alcohol (MBA) as described (Tressel & Kosman, 1980b), except that 1 mg/mL albumin was added to minimize galactose oxidase adsorption to the cuvette. A unit of galactose oxidase activity was defined as 1 nmol of MBA oxidized/min at 25 °C. Dog kidney (Na,K)ATPase was purified by the method of Jørgensen (1974). Eel electroplax microsomes and purified (Na,K)ATPase were prepared by the method of Dixon & Hokin (1978). All preparations were frozen in liquid nitrogen and stored at −70 °C until use. (Na,K)ATPase activity was measured by a coupled enzyme assay at 37 °C as previously described (Moczydlowski & Fortes, 1981b). The protein concentration was determined by quantitative amino acid analysis as previously described (Moczydlowski & Fortes, 1981a) and by a modified Lowry method (Markwell et al., 1978) after deoxycholate–trichloroacetic acid precipitation (Bensadoun & Weinstein, 1976) using an albumin standard calibrated by amino acid analysis. The latter method underestimated (Na,K)ATPase protein concentrations by 10%, whereas in previous work the Lowry method was found to overestimate (Na,K)ATPase concentration up to 40% (Moczydlowski & Fortes, 1981a). This discrepancy is probably related to the use of the deoxycholate–trichloroacetic acid precipitation step in the present work. The variability of (Na,K)ATPase protein determinations with the Lowry method underlines the need to calibrate this procedure when quantitative protein estimates are important. No differences were observed between the Lowry color development of lucifer-labeled preparations and control preparations. All (Na,K)ATPase protein concentrations were corrected for the underestimation by the Lowry method.

(Na,K)ATPase Labeling Procedure. Purified dog kidney (Na,K)ATPase (1 mL of 2–4 mg/mL) was incubated with 60–120 units of galactose oxidase, 1.4–2.8 units of neuraminidase, and 2000–4000 units of catalase in 50 mM Hepes–NaOH/1 mM EDTA, pH 7.5, for 2 h at 0 °C. The reaction was stopped by the addition of 5 mM DTT. The pH was decreased to 6.3 by addition of 1 M succinate/2 mM EDTA, pH 5.9, the hydrazide was added at the final concentrations stated in the figure legends, and the incubation was continued at 0 °C in the dark. The lucifer yellow CH concentration was 4.5 mM unless otherwise stated. After various lengths of time, the pH was raised to 7.4 with 1 M Hepps/2 mM EDTA, pH 8.0; 25 mM $NaBH_4$ was added, and the incubation was continued for 30 min at 0 °C. In some preparations, the hydrazones were reduced with $NaBH_3CN$ at pH 6.3 under the conditions stated in Table III. Reduction with $NaBH_4$ was preferred since this reagent reduces both hydrazones and aldehydes and therefore eliminates the possibility of protein cross-linking. The labeled (Na,K)ATPase was separated from excess probe by centrifugation through a discontinuous sucrose gradient (37%, 28%, and 15%) in an SW-41 rotor for 3 h at 35000 rpm. The enzyme was removed from the 28–37% interface, washed twice by sedimentation and resuspension in 50 mM Mops–Tris/1 mM EDTA, pH 7.0, and then resuspended in an identical buffer containing 30% (w/v) glycerol. The preparation was divided into aliquots, frozen in liquid nitrogen, and stored at −70 °C until use.

Preparation and Labeling of Red Blood Cell Ghosts. White erythrocyte ghosts were obtained by hypotonic lysis of recently outdated human red cells (Dodge et al., 1963). The ghosts were washed twice in 135 mM NaCl/5 mM KCl/10 mM KH_2PO_4, pH 7.4 (PBS 7.4; PBS 8.0 had the same concentration of salts, pH 8.0). Periodate oxidation was performed as previously described (Gahmberg & Andersson, 1977). Equal volumes of packed ghosts and 4.2 mM KIO_4 in PBS 7.4 were mixed at 0 °C in the dark. After 15 min, the oxidation reaction was quenched by dilution in 3 volumes of 100 mM glycerol in PBS 7.4. The ghosts were then washed twice with glycerol–PBS 7.4 and 3 times with 100 mM succinate, pH 6.0, and resuspended in an equal volume of succinate buffer. The ghost suspension was incubated with fluorescent amines or hydrazides (final concentration between 1 and 13 mM) for 2 h at 37 °C. The suspensions were cooled to 0 °C and incubated for 40 min with an equal volume of 50 mM $NaBH_4$ in PBS 8.0. The labeled ghosts were washed with PBS 8.0 and prepared for SDS–PAGE.

SDS–Polyacrylamide Gel Electrophoresis. Two-millimeter-thick slab gels were formed by the Laemmli (1970) procedure. The samples were dissolved in 2% SDS, made 25 mM in DTT, and incubated at 37 °C for 1 h before addition to the gel. After electrophoresis, a photograph of the gel fluorescence was taken under ultraviolet illumination with a Polaroid camera equipped with a 1 cm, 1% (w/v) K_2CrO_4 filter and Kodak Wratten 15 and 58 filters. The gel in Figure 2 was prerun roughly 33% of its length before the samples were loaded. Prerunning eliminated fluorescent gel contaminants which otherwise collect at the gel front. The gels were fixed in methanol–acetic acid–water (10:1:9), stained with Coomassie Blue R-250, and destained in the fixing solution.

Probe Synthesis. All steps were performed in the dark or under dim light. The components of the reaction mixture were separated by TLC. The products were identified by their fluorescence, their altered R_f after reaction with acetone (indicating the presence of a functional group which reacts with carbonyl groups), and their ninhydrin reactivity (indicating the presence of amino groups).

5-[(Dicarbohydrazidotriazinyl)amino]fluorescein (DCTAF). 5-[(Dichlorotriazinyl)amino]fluorescein (DTAF) in ethanol was added dropwise (final concentration 5 mM)

to 0.124 M aqueous carbohydrazide at 70 °C and incubated for 24 h. The reaction mixture was dried by rotoevaporation, redissolved in chloroform–methanol–acetic acid (2:1:0.05), and centrifuged to clarify. The supernatant was loaded on a 2.5 cm × 60 cm Biosil A column and eluted with the same acidic chloroform–methanol mixture. The column separated the reaction mixture into three fluorescent bands. After elution of the first two bands with chloroform–methanol–acetic acid (2:1:0.05), the product was eluted with alkaline methanol and evaporated to dryness.

Since DTAF is a bifunctional reagent, mono- or disubstituted adducts could be formed. The product described above appears to be the dicarbohydrazide derivative rather than the monosubstituted derivative for the following reasons. (1) When the reaction mixture described above was incubated at room temperature for 10 h, a fluorescent product (P1) was formed [R_f = 0.27 silica TLC plates developed in chloroform–methanol–acetic acid (4:1:0.1)]. P1 reacted with acetone and ninhydrin and therefore contained the carbohydrazide group. (2) When this incubation was continued for 2 days at room temperature, a second fluorescent product (P2) appeared. P2 also reacted with acetone and ninhydrin. (3) P1 was completely converted to P2 after the reaction mixture was incubated further for 21 h at 70 °C. P2 did not migrate on TLC plates developed in chloroform–methanol–acetic acid (4:1:0.1, 3:1:0.05, and 2:1:0.05) but reacted with acetone to form a single fluorescent product which migrated on TLC plates developed in chloroform–methanol–acetic acid (2:1:0.05, R_f = 0.56; 3:1:0.05, R_f = 0.34; and 4:1:0.1, R_f = 0.29). (4) Only P2 was detected after 24-h incubation at 70 °C.

The sequential formation of P1 and P2 at room temperature and the accelerated formation of P2 without detectable formation of P1 at 70 °C are consistent with the known, temperature-dependent, stepwise substitution of the chlorine atoms of 2,4,6-trichlorotriazine (cyanuric chloride) by amines, substituted amines, and hydrazides (Thurston et al., 1951; Kaiser et al., 1951; Beech, 1967). The stepwise substitution can be attributed to the lower reactivity of the remaining chlorine atom of DTAF by analogy to the differences in reactivity of cyanuric chloride compared to 2-amino-4,6-dichlorotriazine and 2,4-diamino-6-chlorotriazine (Fries, 1886; Diels, 1899; Mosher & Whitmore, 1945). These data suggest that P1 is the monocarbohydrazide adduct and P2 is the dicarbohydrazide adduct of DTAF.

N-Dansylcarbohydrazide. A methanol solution of 1,5-dansyl chloride was added dropwise (10 mM final concentration) to aqueous 0.124 M carbohydrazide at 65 °C, incubated for 6 h, cooled to room temperature, and stored at −20 °C. The resulting crystals were filtered, washed with ice-cold methanol, dried under vacuum at room temperature, and stored at −20 °C. *N*-Dansylcarbohydrazide reacted with ninhydrin and migrated as a single species on silica TLC plates developed in butyl acetate–methanol (3:1) before (R_f = 0.47) and after acetone treatment (R_f = 0.68). The reactivity with ninhydrin and acetone indicates the presence of the carbohydrazide functional group in the product.

Probe concentrations were determined spectrophotometrically with the following molar absorption coefficients: anthraniloylhydrazide, 3640 M^{-1} cm^{-1} at 325 nm (Sadtler spectrum 14303 UV); 5-aminofluorescein, 78 800 M^{-1} cm^{-1} at 494 nm (Sadtler spectrum 11447 UV); anthracene-9-carbohydrazone, 3640 M^{-1} cm^{-1} at 325 nm; pyrenebutyrylhydrazide, 36 500 M^{-1} cm^{-1} at 348 nm; proflavin monosemicarbazide, 4600 M^{-1} cm^{-1} at 445 nm (Reines & Cantor, 1974); lucifer yellow CH, 11 900 M^{-1} cm^{-1} at 428 nm (Stewart, 1981); 5-[(dichlorotriazinyl)amino]fluorescein and its carbohydrazide derivative, 82 000 M^{-1} cm^{-1} at 495 nm (Barskii et al., 1968); eosinthiosemicarbazide, 80 000 M^{-1} cm^{-1} at 525 nm; fluoresceinthiosemicarbazide, 70 000 M^{-1} cm^{-1} at 495 nm; dansyl derivatives, 4500 M^{-1} cm^{-1} at 340 nm (Haugland, 1981).

Fluorescence Measurements. Anthroylouabain titrations and binding kinetics were conducted at 37 °C as previously described (Fortes, 1977; Moczydlowski & Fortes, 1980). The semilogarithmic plots of the kinetics of AO association and dissociation were linear over more than 90% of the approach to equilibrium.

The steady-state anisotropy of lucifer-labeled (Na,K)ATPase was measured at 25 °C with excitation at 430 nm through a Corning 5-60 filter and emission at 540 nm through a Corning 3-70 filter and was calculated as described (Dale & Eisinger, 1975). The anisotropy was corrected, with horizontally polarized excitation, for instrumental polarization; all measurements were corrected for light scattering and blank fluorescence.

The lucifer content of labeled (Na,K)ATPase was determined after washing by comparing its fluorescence intensity with that of a lucifer yellow CH fluorescence standard curve. The assumption of identical quantum yields of lucifer-labeled (Na,K)ATPase and free lucifer CH was justified by control experiments which showed that lucifer yellow CH did not change spectral shape, excitation and emission maxima, or fluorescence intensity (≤3%) after reaction with acetone (hydrazone formation) or hydrazone reduction with $NaBH_4$. The moles of lucifer per mole of β were estimated from the lucifer content per milligram of total protein, assuming the enzyme was 100% pure and the molecular weight of $\alpha\beta$ = 180 000 (Craig & Kyte, 1980).

The relative amount of lucifer labeling in Figure 2 was estimated by photographing the gel fluorescence and integrating the microdensitometer scan of the photographic negative (Jesaitis & Fortes, 1980).

Results and Discussion

To develop a labeling technique specific for β that would not alter the structure or function of (Na,K)ATPase, a survey of oligosaccharide oxidation methods and fluorescent labeling reagents was undertaken. The (Na,K)ATPase labeling strategy consists of (1) oxidation of β-subunit oligosaccharides, (2) reaction of the resulting aldehyde groups with fluorescent hydrazides, (3) reduction of the fluorescent hydrazones and unreacted aldehydes with $NaBH_4$, and (4) separation of the labeled enzyme from the excess hydrazide (Figure 1). The oxidation method and fluorescent reagent used for labeling were found to influence the labeling specificity and inactivation of (Na,K)ATPase.

Enzymatic Oxidation. Galactose oxidase was studied as an oxidation reagent since enzymatic techniques should be more selective and potentially less inhibitory than chemical methods. (Na,K)ATPase was incubated in the presence and absence of neuraminidase and galactose oxidase, incubated with lucifer yellow CH, and washed as described under Materials and Methods. The gel stained with Coomassie Blue (Figure 2B) shows the (Na,K)ATPase α and β subunits as well as contaminating proteins. Neuraminidase treatment increased the mobility of β (Figure 2B). This was probably due to a decrease in molecular weight and an increase in the charge to protein mass ratio caused by increased SDS binding following cleavage of sialic acid residues (Segrest & Jackson, 1972). A single fluorescent band, corresponding to β, was observed only in samples incubated with galactose oxidase

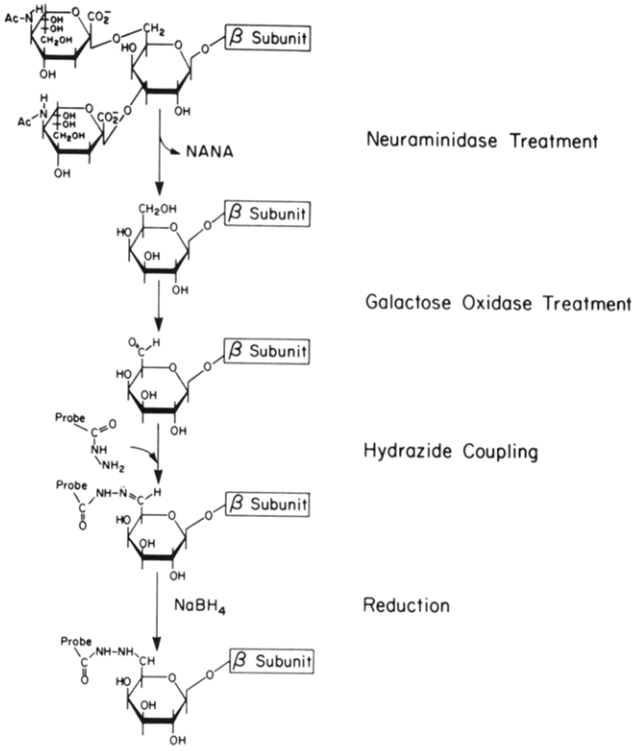


FIGURE 1: Scheme of the β-subunit labeling procedure.

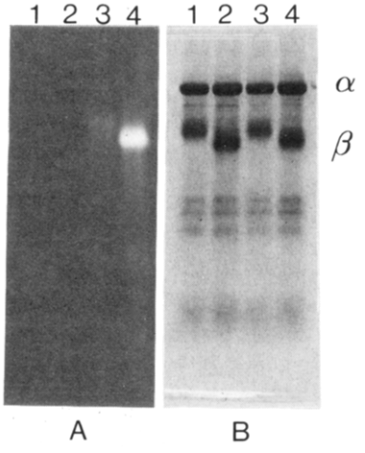


FIGURE 2: Effect of galactose oxidase and neuraminidase treatment on fluorescent labeling of (Na,K)ATPase. (Na,K)ATPase was incubated in the presence or absence of galactose oxidase and neuraminidase, treated with lucifer yellow CH, reduced, washed, and submitted to SDS–PAGE as described under Materials and Methods. Photograph of the unstained gel fluorescence (A) and the gel after Coomassie Blue staining (B). Lane 1, no galactose oxidase or neuraminidase addition; lane 2, neuraminidase; lane 3, galactose oxidase; lane 4, neuraminidase and galactose oxidase. The positions of the α and β subunits are indicated; 55 μg of protein was applied to each lane.

(Figure 2A); the absence of other fluorescent bands indicates that the labeling was specific for β. Neuraminidase treatment alone was not sufficient for labeling. Simultaneous neuraminidase and galactose oxidase treatment increased the extent of labeling 7-fold when compared to treatment with galactose oxidase alone.

The following evidence suggests that oxidized galactose residues of β-subunit oligosaccharides were required for (Na,K)ATPase labeling. The β subunit, a glycoprotein, was specifically labeled although α and other contaminating polypeptides were present. Labeling had an absolute requirement for galactose oxidase, which oxidizes to an aldehyde the C_6 hydroxyl of galactose and oligosaccharides containing galactose (Avigad et al., 1962; Amaral et al., 1966). The increased labeling of β with neuraminidase is consistent with the enzymatic removal of sialic acid residues and exposure of penultimate galactose residues (Figure 1). Reduction with $NaBH_4$ following galactose oxidase plus neuraminidase treatment prevented labeling of β by lucifer yellow CH; this demonstrates that glycoprotein carbonyl groups were required for labeling.

Periodate Oxidation. Periodate oxidation of oligosaccharides was an alternative to the galactose oxidase method (Abraham & Low, 1980; Cherry et al., 1980). Oxidations under various KIO_4 concentrations, buffer compositions, temperatures, pHs, and incubation times inhibited (Na,K)-ATPase activity 50–80% under conditions which resulted in low or undetectable labeling. Periodate also inactivates ovotransferrin and human serum transferrin (Geoghegan et al., 1980). Enzyme inactivation is not unexpected since periodate oxidizes amino-terminal serine, threonine, and cysteine to produce α-oxoacyl derivatives (Dixon & Weitkamp, 1962; Clamp & Hough, 1965; Fields & Dixon, 1968) and destroys several amino acids in solution and cysteine, methionine, tryptophans, and tyrosines in proteins (Clamp & Hough, 1965; Knowles, 1965; Astassi, 1967; Gan et al., 1968). In addition, oxidation of purified (Na,K)ATPase and microsomes from dog kidney or electric eel with 2 mM periodate caused polypeptide cross-linking as detected on SDS–PAGE. Polypeptide cross-linking has been observed after periodate treatment of *Torpedo marmorata* membranes and detergent-solubilized acetylcholine receptor (Criado & Barrantes, 1982) but was not observed with human red blood cells (Abraham & Low, 1980).

Probe Selection. Several fluorescent probes were surveyed in an attempt to label β specifically without perturbing the structure or activity of the enzyme. Of the compounds tested, five probes (Figure 3) labeled (Na,K)ATPase treated with galactose oxidase and neuraminidase. Figure 4 shows the labeling pattern obtained with these probes.

Lucifer yellow CH labeled β specifically (Figure 2 and Figure 4, lanes 7 and 8). Nonspecific adsorption of lucifer yellow CH was eliminated by centrifugation through a discontinuous sucrose gradient and washing as indicated by the absence of fluorescent material at the gel front (Figure 2A, lanes 3 and 4). The anisotropy of lucifer-labeled (Na,K)-ATPase did not change significantly after 4 h at 25 °C or after several days at 0 °C and indicated that the lucifer–β adduct was stable.

(Na,K)ATPase was also labeled by DCH, DCTAF, FTC, and PBH (Figure 4); however, these probes exhibited other properties which make them less suitable for spectroscopic studies. PBH labeled β extensively (Figure 4A, lane 2); however, PBH, probably because it is hydrophobic, adsorbed to the membrane and could not be removed by dialysis, centrifugation, or treatments with SM-2 Biobeads and defatted albumin. PBH may be useful to label soluble glycoproteins or membrane glycoproteins prior to detergent solubilization and fractionation or when nonspecific adsorption is unimportant. DCH labeled β specifically (Figure 4A, lane 5), but both aqueous solutions of the free probe and labeled (Na,-K)ATPase were unstable. Upon illumination, the DCH emission maximum shifted from 570 to 510 nm concomitant with the production of 1,5-dansic acid. Photochemical hydrolyses of dansyl amino acids (D'Souza et all., 1970) and dansylhydrazine adducts (Gregorio Weber, personal communication) have been noted previously. The photochemical instability of DCH and its glycoprotein adduct limits the usefulness of this probe. FTC labeled β (Figure 4A, lane 6);

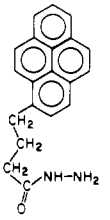
Table I: Fluorescent Labeling of Periodate-Treated Red Cell Ghosts and (Galactose Oxidase + Neuraminidase)-Treated (Na,K)ATPase

probe	(Na,K)ATPase[a]	ghosts[b]
anthraniloylhydrazide	−	+
anthracene-9-carbohydrazone	−	+
5-aminofluorescein	−	+
N-dansylcadaverine	±	nd
N-dansylethylenediamine	±	+
eosinthiosemicarbazide	−	+
ethidium monosemicarbazide	−	+
proflavin monosemicarbazide	−	+

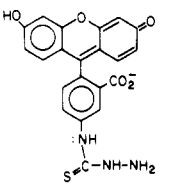
[a] (Na,K)ATPase was treated with galactose oxidase and neuraminidase as described in the text and diluted with either 100 mM succinate–3 mM EDTA, pH 6.0, or 50 mM Pipes–Tris/3 mM EDTA, pH 7.5, or 100 mM borate–3 mM EDTA, pH 9.0, to adjust the pH to 6.4, 7.5, and 8.4, respectively. The specified fluorescent reagent was added to a final concentration of 1.0–12.7 mM, incubated 13–57 h at 0 °C, sedimented, and analyzed by SDS–PAGE. Faint labeling was observed with *N*-dansylcadaverine and *N*-dansylethylenediamine after 57-h and 16-h incubation at pH 8.4, respectively. [b] Periodate oxidation and fluorescent labeling of ghosts were carried out as described under Materials and Methods. (+) denotes labeling; (−) denotes no labeling was detected.

occasionally, however, labeling of α and protein cross-linking were observed. Since fluorescein isothiocyanate readily labels α (Karlish, 1980), the FTC labeling of α may be due to contaminating fluorescein isothiocyanate remaining from the FTC synthesis. Contaminating hydrazine may explain the FTC-mediated cross-linking. In addition, FTC (or contaminating fluorophores) adsorbed to the membrane and could not be removed by dialysis, centrifugation, or treatments with SM-2 Biobeads and defatted albumin. DCTAF labeled approximately 10% of the β subunits without cross-linking when the extent of oxidation was limited (Figure 4A, lane 4); however, when the labeling stoichiometry was increased with the standard oxidation procedure, extensive cross-linking of β subunits by DCTAF was observed (Figure 4, lane 3). DCTAF probably induced β-subunit cross-linking due to its bifunctional nature. Previously, α–α and β–β cross-linkings have been used to suggest that (Na,K)ATPase is composed of $\alpha\beta$ oligomers (Kyte, 1975; Giotta, 1976; Askari et al., 1980); however, the structural significance of cross-linking in the study of membrane proteins is uncertain because of the long reaction times as well as the high concentration and the rapid lateral diffusion of membrane-bound proteins which may lead to cross-linking of unassociated molecules during their collisions (Kyte, 1981). DCTAF may be a useful fluorescent cross-linking reagent, but its utility is limited in studies requiring a native quaternary enzyme structure.

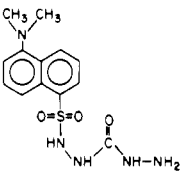
Several fluorescent amines and hydrazides labeled human erythrocyte ghosts oxidized by periodate but did not label (Na,K)ATPase treated with neuraminidase and galactose oxidase (Table I). The ghosts were oxidized at 0 °C as described by Gahmberg & Andersson (1977). This method preferentially oxidizes the exocyclic portion of sialic acids and results in an aldehyde product structurally similar to the



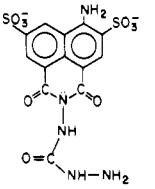
Pyrene butyrylhydrazide



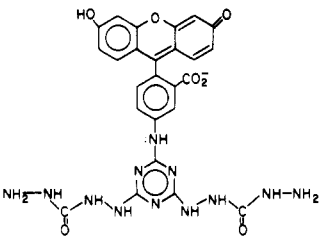
Fluorescein-thiosemicarbazide



Dansyl carbohydrazide



Lucifer Yellow carbohydrazide



Dicarbohydrazidotriazinylamino Fluorescein

FIGURE 3: Structures of fluorescent hydrazides for (Na,K)ATPase β-subunit labeling.

product generated by galactose oxidase treatment (Figure 1). The reason for the difference in reactivity of these probes with (Na,K)ATPase and erythrocyte glycoproteins is not clear. The lack of reactivity in (Na,K)ATPase may reflect steric hindrance or inaccessibility to the reagent although the structures of some of the reactive (Figure 3) and unreactive (Table I) probes are similar. Therefore, no clear pattern of reactivity can be derived from the available information.

Biochemical Properties of Lucifer-Labeled (Na,K)ATPase. To see whether or not the labeling procedure perturbed (Na,K)ATPase function, AO binding and (Na,K)ATPase activity were studied. Table II lists the AO kinetic constants and the derived dissociation constants of lucifer-labeled (Na,K)ATPase (62.2 nmol of lucifer/mg of protein) and untreated enzyme. Lucifer labeling did not alter significantly the AO association or dissociation rate constants when binding was promoted by either Mg + P_i or Mg·ATP + Na. AO binding under these ligand conditions reflects phosphorylation of the α subunit (Fortes, 1977; Moczydlowski & Fortes, 1980; Fortes & Lee, 1984); therefore, these results indicate that Mg,

Table II: Kinetic Constants of AO Binding to Lucifer-Labeled and to Untreated (Na,K)ATPase[a]

ligands added	enzyme treatment	k_{on} ($\times 10^4$ M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})	K_d ($=k_{off}/k_{on}$) (nM)
MgATP + Na[b]	untreated	2.17 (2.15–2.18)	2.38 (2.20–2.55)	11.0
	lucifer	1.91 (1.85–1.97)	2.94 (2.32–3.88)	15.4
Mg + P_i[c]	untreated	2.36 (1.83–2.89)	1.34 (1.00–1.67)	5.6
	lucifer	2.51 (2.29–2.72)	1.35 (1.03–1.67)	5.4

[a] Pseudo-first-order AO binding was measured at 37 °C in 50 mM Pipes–Tris, pH 7.0, to lucifer-labeled (Na,K)ATPase (62.2 nmol of lucifer/mg of protein, approximately 11 mol of lucifer/mol of β subunit) or a control sample. AO dissociation was measured after addition of 150 μM ouabain. The k_{on} and k_{off} values are the means of duplicate or triplicate determinations; the ranges are indicated in parentheses. [b] The incubation mixture contained 20 μM ATP, 17 mM $MgCl_2$, 26 mM NaCl, 10 mM Tris–CDTA, 8.3 mM $(Tris)_2$–phosphocreatine, 4.8 units/mL rabbit muscle creatine kinase, 99 nM (Na,K)ATPase, and 1.32 μM AO. [c] The incubation mixture contained 13 mM $MgCl_2$, 6 mM Tris–P_i, 10 mM Tris–CDTA, 99 nM (Na,K)ATPase, and 1.32 μM AO.

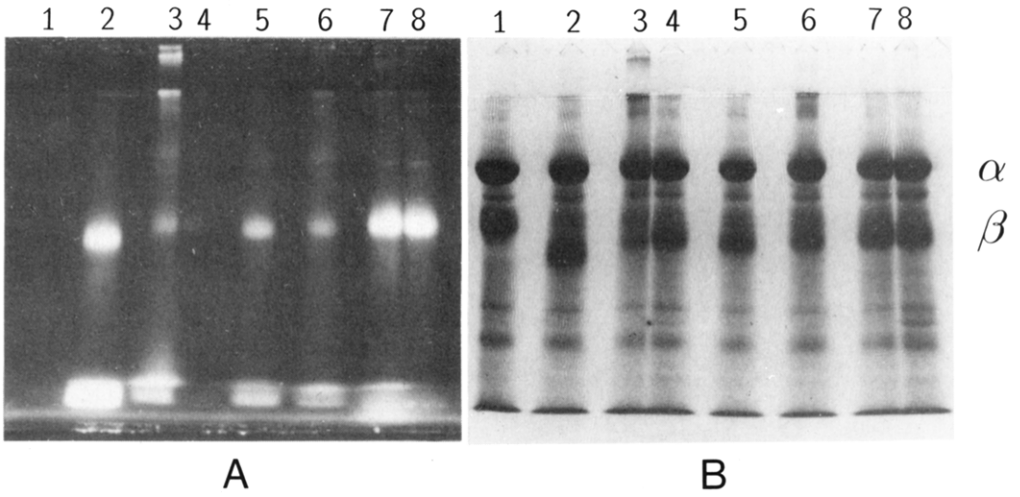


FIGURE 4: Labeling of the (Na,K)ATPase β subunit by different fluorescent probes. Photograph of fluorescence of the unfixed gel (A) and of the gel after Coomassie Blue staining (B). The positions of the α and β subunits are indicated; 75 μg of protein was applied to each lane. (Na,K)ATPase was oxidized as described under Materials and Methods, except for lane 1, which was not treated with neuraminidase and galactose oxidase. The pH was decreased to 6.4, and the oxidized enzyme was incubated in the presence of the following hydrazides for 24 h at 0 °C: lane 2, 2.3 mM PBH; lane 3, 0.9 mM DCTAF; lane 4, 0.9 mM DCTAF and oxidation was with neuraminidase and 15–30 units of galactose oxidase for 1 h at 0 °C to reduce the extent of oxidation; lane 5, 3 mM DCH; lane 6, 1 mM FTC; lanes 7 and 8, 4 mM lucifer yellow CH. Reduction with $NaBH_4$ was carried out at pH 7.4 as described under Materials and Methods. Samples 1–7 were diluted and sedimented once; sample 8 was washed as described under Materials and Methods. The fluorescent material at the bottom of the gel included both gel contaminants and unreacted probe which adsorbed to the membrane.

ATP, P_i, and Na binding, as well as the conformational changes associated with phosphorylation and the properties of the cardiac glycoside site, was not affected by the labeling procedure.

Anthroylouabain titrations of untreated, oxidized–reduced, and lucifer-labeled (Na,K)ATPase preparations showed a high-affinity, saturable enhancement of AO fluorescence (Figure 5), indicative of AO binding to the ouabain site (Fortes, 1977). The high-affinity equilibrium binding of AO is in agreement with the dissociation constants of about 5 nM estimated from the kinetic measurements (Table II) and allowed the determination of the number of AO binding sites from the intersection of the lines drawn through the points below and above saturation (Figure 5). Since the concentration of sites was ≥20 times the AO dissociation constant, AO binding was essentially stoichiometric below ≃80% saturation. Therefore, the initial slopes of the titrations are proportional to the quantum yield of bound AO. The initial slopes of the titrations were identical for the untreated and oxidized–reduced preparations (9.5 times greater than that of free AO in buffer), but the lucifer-labeled enzyme showed a smaller slope (Figure 5). When the titrations of (Na,K)-ATPase preparations labeled to different extents with lucifer were compared, it was seen that the initial slope decreased when the lucifer stoichiometry increased (not shown). These results indicate that the quantum yield of specifically bound AO, which monitors the environment near the ouabain site (Fortes, 1977), was unaltered by treatment with galactose oxidase plus neuraminidase and reduction with $NaBH_4$. The decreased quantum yield of specifically bound AO in the lucifer-labeled samples probably reflects resonance energy transfer from AO to lucifer, since the lucifer excitation spectrum (see below) overlaps the AO emission spectrum. This result suggests that the sites of lucifer attachment on β are within energy transfer distance from the site of AO binding on α. A detailed study of AO to lucifer energy transfer will be presented elsewhere.

Table III shows the lucifer labeling stoichiometries, the (Na,K)ATPase activities, and the number of AO sites of various preparations. The amount of lucifer incorporation could be controlled by varying the temperature or the duration of the reaction with lucifer. The labeling procedure had no significant effect on either the (Na,K)ATPase activity or the number of AO sites. The small inhibitions observed in some preparations were seen also when the enzyme was oxidized and

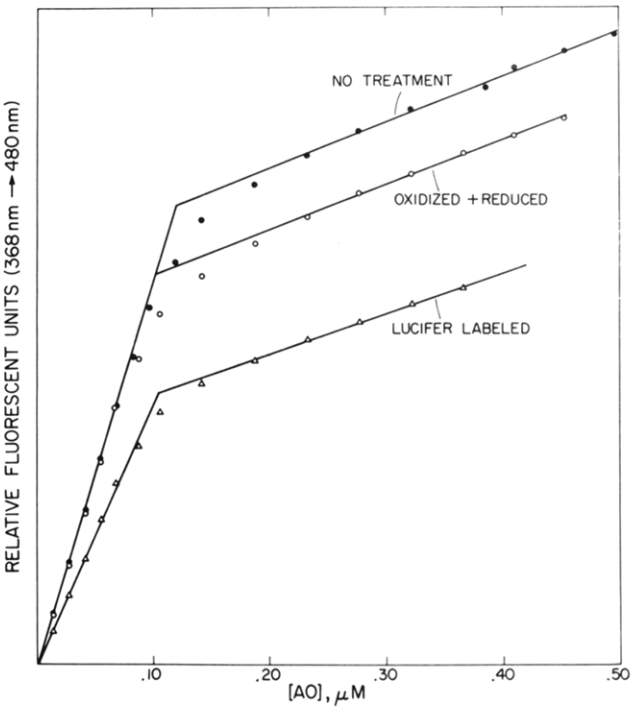


FIGURE 5: Anthroylouabain titrations. AO titrations were conducted in 3.3 mM phosphate–Tris/6.7 mM $MgCl_2$, 50 mM Mops–Tris (pH 7.0), and 1 mM Tris–EDTA with 30 μg of protein/mL of oxidized–reduced (○), or lucifer-labeled (△), or untreated (Na,K)ATPase (●). The temperature was 37 °C. Sequential AO additions were made, and the fluorescence of AO at equilibrium was plotted. Since AO binding was essentially stoichiometric under these conditions, the initial slopes of the titrations are proportional to the quantum yield of specifically bound AO, and the intersection of the lines drawn through the points at low and high [AO] gives the following concentrations of AO binding sites: 120 nM (untreated); 102 nM (oxidized–reduced); and 108 nM (lucifer labeled).

Table III: Effect of Lucifer Labeling on (Na,K)ATPase Activity and AO Site Stoichiometry[a]

expt	treatment	lucifer stoichiometry: nmol/mg	lucifer stoichiometry: mol of lucifer/mol of β[b]	(Na,K)-ATPase activity (% of control)	AO sites (nmol/mg)
I[c]	control			100	4.2
	labeled (3 h)	8.5	1.5	79	4.8
	labeled (21 h)	25.6	4.6	70	4.6
II	control			100	4.5
	labeled[d] (3 h)	6.4	1.1	92	4.7
	labeled[e] (3 h)	9.7	1.7	84	4.7
	labeled[f] (3.5 h)	5.8	1.0	89	4.5
III[g]	control (frozen)			100	
	labeled (1 h)	5.4	1.0	102	
	labeled (3 h)	10.3	1.9	97	
	labeled (5 h)	14.9	2.7	108	
	labeled (8.3 h)	19.9	3.6	110	
	labeled (14 h)	25.5	4.6	98	
	labeled (25 h)	32.3	5.8	127	
IV	control[h] (frozen)			100	4.0
	oxidized–reduced[i]			76	3.4
	labeled	62.2	11.0	76	3.6

[a] (Na,K)ATPase was treated with neuraminidase and galactose oxidase and reduced with $NaBH_4$ as described under Materials and Methods except where indicated otherwise. The enzyme suspension was incubated with 4.5 mM lucifer for the indicated time at 0 °C. The labeled enzyme was separated from unreacted probe as described in the text, resuspended, and frozen in liquid nitrogen. (Na,K)ATPase activity and lucifer stoichiometry were determined as described under Materials and Methods. The AO site stoichiometry was determined as in Figure 5. Protein concentrations, (Na,K)ATPase activities, and lucifer stoichiometries represent the means of duplicate or triplicate determinations. Each AO site stoichiometry is the result of a single determination. Control samples were not oxidized, reduced, or frozen unless otherwise noted. The specific activity of the untreated enzyme was 27–30 μmol min^{-1} (mg of protein)$^{-1}$. [b] Estimated by assuming the enzyme was 100% pure and the molecular weight of $\alpha\beta$ is 180000. [c] $NaBH_3CN$ (40 mM) was included in the incubation with lucifer; at the indicated times, the labeled enzyme was separated from the excess probe without further reduction. [d] After 3-h incubation with lucifer, 15 mM $NaBH_3CN$, pH 6.3, was added, and the incubation was continued for 30 min at 0 °C before washing. [e] After 3-h incubation with lucifer, 15 mM $NaBH_3CN$, pH 6.3, was added, and the incubation was continued for 30 min at 37 °C before washing. [f] $NaBH_3CN$ (15 mM) was included during the incubation with lucifer; the sample was washed after 3.5 h. [g] Aliquots were withdrawn at the indicated times, adjusted to pH 7.4 with 1 M Hepps and 2 mM EDTA, pH 8.0, and frozen in liquid nitrogen to stop the labeling. Each aliquot was thawed, incubated with $NaBH_4$, and washed by centrifugation. The control enzyme was diluted to the same concentration as that of the labeled enzyme and was frozen in liquid nitrogen; after freeze–thawing, its activity decreased to 71% of that of an unfrozen sample. [h] The control was resuspended in buffered glycerol, pH 7.0, and frozen. [i] This sample was oxidized and reduced with $NaBH_4$ without incubation with lucifer.

reduced without the addition of lucifer (Table III) and when untreated (Na,K)ATPase was diluted in buffer, incubated at 0 °C, frozen, and thawed prior to protein and activity determinations, as was done with the lucifer-labeled preparations. In addition, the decreased activity of some preparations was not accompanied by a decrease in the number of AO sites (Table III). Therefore, we suspect that the differences in activity between labeled and untreated enzyme reflect inactivations by freeze–thawing and propagation of experimental errors in the determinations of protein and activity, and are unrelated to the labeling procedure.

Spectroscopic Properties of Lucifer Yellow CH and Lucifer-Labeled (Na,K)ATPase. Figure 6 shows the corrected emission spectra of lucifer yellow CH in various solvents. The fluorescence intensity and emission maximum were solvent dependent. The intensity increased and the emission maximum

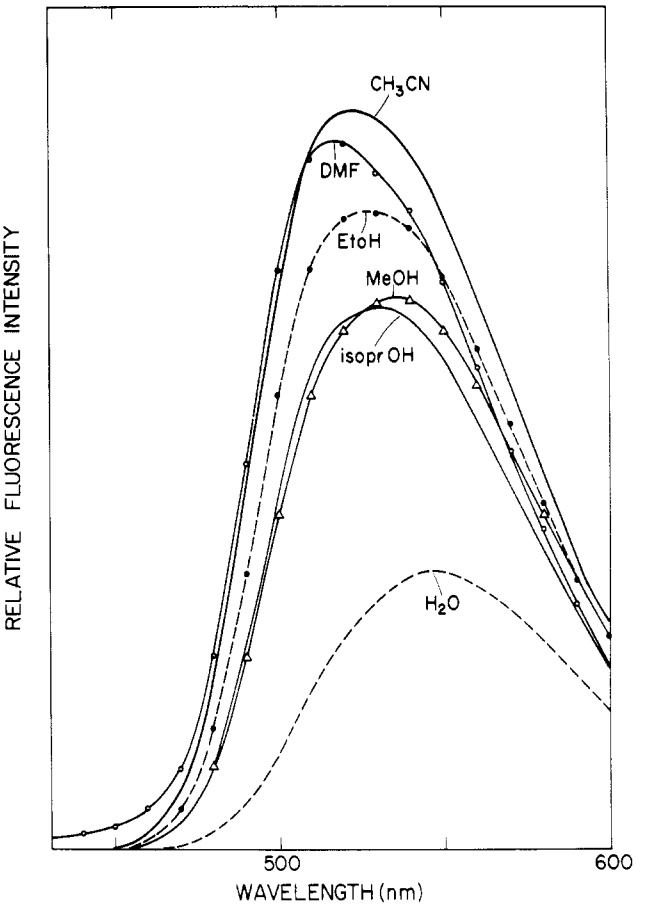


FIGURE 6: Effect of solvents on lucifer yellow CH emission spectra. Corrected emission spectra of 0.44 μM lucifer yellow CH in acetonitrile (CH_3CN), dimethylformamide (DMF), ethanol (EtOH), methanol (MeOH), isopropyl alcohol (isoprOH), and water (H_2O) were recorded. The excitation wavelength was 410 nm with 10- and 5-nm band-pass values for excitation and emission, respectively.

shifted to the blue in solvents less polar than water. The solvent dependence of the fluorescence intensity was due primarily to a change in quantum yield since the absorbance of lucifer solutions in ethanol and water varied by less than 10%.

Corrected excitation and emission spectra of lucifer-labeled (Na,K)ATPase showed excitation and emission maxima at 432 ± 2 and 544 ± 1 nm, respectively (Figure 7). The emission maximum of lucifer-labeled (Na,K)ATPase was identical with the emission maximum of lucifer in aqueous solution (Figure 6) and suggested that the sites of lucifer attachment on β are in an aqueous environment.

The fluorescence anisotropy of lucifer-labeled (Na,K)ATPase was 0.14, significantly different from the anisotropy of lucifer yellow CH in aqueous solution (0.02) and the anisotropy of an immobilized fluorophore, theoretically 0.4. This indicates that the attachment of lucifer yellow CH to β-subunit oligosaccharides restricts the motion of the probe but does not immobilize it totally. Since the (Na,K)ATPase molecules are embedded in a lipid bilayer, rotational and translational motion of the whole protein is negligible in the nanosecond time scale. Therefore, the motional freedom of lucifer in the labeled (Na,K)ATPase probably reflects segmental flexibility of the oligosaccharide chains and/or rotation of the probe around its point of attachment.

Conclusions

This work describes a method to label the (Na,K)ATPase β subunit with fluorescent hydrazides; the method should be a general labeling technique for glycolipids and glycoproteins

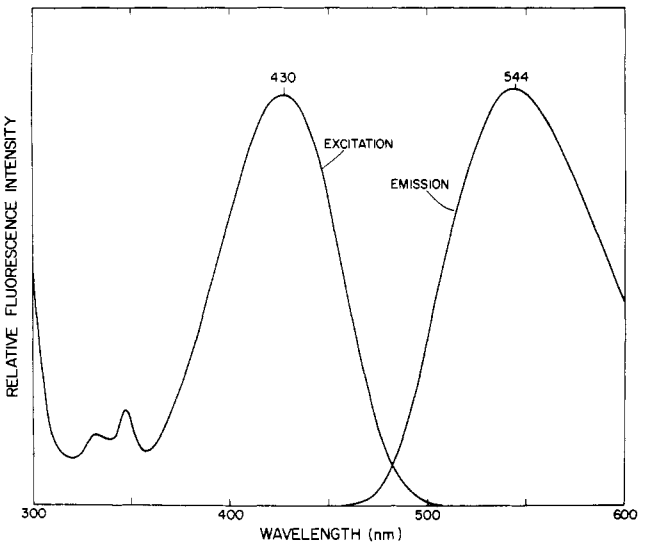


FIGURE 7: Fluorescence spectra of lucifer-labeled (Na,K)ATPase. Corrected excitation and emission spectra of lucifer-labeled (Na,-K)ATPase (62 nmol of lucifer/mg of protein, 44 μg of protein/mL) in 50 mM Mops–Tris/1 mM EDTA, pH 7.0, 25 °C, were recorded. The excitation and emission wavelengths were 430 and 550 nm for the emission and excitation spectra, respectively.

containing galactose. Several fluorescent hydrazides labeled β in (Na,K)ATPase treated with neuraminidase plus galactose oxidase; among these, only lucifer yellow CH formed stable adducts with β and did not induce cross-linking, and its nonspecific adsorption was easily reversed by washing. The remaining reagents may be useful in labeling glycoproteins prior to purification or for use in nonspectroscopic studies.

Lucifer labeling was specific for the oligosaccharides of β. Labeling stoichiometries up to approximately 11 mol of lucifer/mol of β were obtained without significant (Na,K)ATPase inactivation or perturbation of the cardiac glycoside site. The fluorescence emission spectrum and anisotropy of lucifer-labeled (Na,K)ATPase suggest that the probe is in an aqueous environment and that the labeled oligosaccharides have significant motional freedom in the nanosecond time scale. The resonance energy transfer observed between AO bound to α and lucifer attached to β suggests that the labeled oligosaccharides are within 70 Å of the cardiac glycoside site.

Fluorescent labeling of β may be utilized for a variety of studies. Labeled β-subunit oligosaccharides should be useful in identifying carbohydrate-containing peptides following fragmentation of β. The environmental sensitivity of lucifer yellow fluorescence may be useful to detect ligand-dependent conformational changes of β, and thus to study the possible role of β in (Na,K)ATPase function. Labeled β-subunit oligosaccharides may serve as a reference point for energy transfer studies in (Na,K)ATPase. Since lucifer-labeled β-subunit oligosaccharides were efficient energy acceptors for AO specifically bound to the cardiac glycoside site on the α subunit, energy transfer studies may provide quantitative information on α–β interactions and their changes under various conformational states.

ACKNOWLEDGMENTS

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Registry No. ATPase, 9000-83-3; DCTAF, 94137-28-7; DCH, 94137-29-8; FTC, 75323-82-9; PBH, 55486-13-0; AO, 62026-02-2; DTAF, 51306-35-5; lucifer yellow CH, 67769-47-5; dansyl chloride, 605-65-2; carbohydrazide, 497-18-7.

REFERENCES

Abraham, G., & Low, P. S. (1980) *Biochim. Biophys. Acta 597*, 285–291.
Amaral, D., Kelly-Falcoz, F., & Horecker, B. L. (1966) *Methods Enzymol. 9*, 87–92.
Askari, A., Huang, W., & Antieau, J. M. (1980) *Biochemistry 19*, 1132–1252.
Atassi, M. Z. (1967) *Arch. Biochem. Biophys. 120*, 56–69.
Avigad, G., Amaral, D., Asensio, C., & Horecker, B. L. (1962) *J. Biol. Chem. 237*, 2736–2743.
Barskii, V. E., Ivanov, V. B., Slkyar, Y. E., & Mikhailov, G. I. (1968) *Izv. Akad. Nauk SSSR, Ser. Biol.*, 744.
Beech, W. F. (1967) *J. Chem. Soc. C*, 466–472.
Bensadoon, A., & Weinstein, B. (1976) *Anal. Biochem. 70*, 241–250.
Brotherus, J. B., Møller, J. V., & Jørgensen, P. L. (1981) *Biochem. Biophys. Res. Commun. 100*, 146–154.
Cantley, L. C. (1981) *Curr. Top. Bioenerg. 11*, 201–237.
Cherry, R. J., Nigg, E. A., & Beddard, G. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5899–5903.
Clamp, J. R., & Hough, L. (1965) *Biochem. J. 94*, 17–24.
Craig, W. S. (1982) *Biochemistry 21*, 5707–5717.
Craig, W. S., & Kyte, J. (1980) *J. Biol. Chem. 255*, 6262–6269.
Criado, M., & Barrantes, F. G. (1982) *Neurochem. Int. 4*, 289–302.
Dale, R. E., & Eisinger, J. (1975) in *Biochemical Fluorescence Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 1, pp 115–280, Marcel Dekker, New York.
Diels, O. (1899) *Ber. Dtsch. Chem. Ges. 32*, 691–702.
Dixon, J. F., & Hokin, L. E. (1978) *Anal. Biochem. 86*, 378–385.
Dixon, H. B. F., & Weitkamp, L. R. (1962) *Biochem. J. 84*, 462–468.
Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119–130.
D'Souza, L, Bhatt, K., Madaiah, M., & Day, R. A. (1970) *Arch. Biochem. Biophys. 141*, 690–693.
Fields, R., & Dixon, H. B. F. (1968) *Biochem. J. 108*, 883–887.
Fortes, P. A. G. (1977) *Biochemistry 16*, 531–540.
Fortes, P. A. G., & Lee, J. A. (1984) *J. Biol. Chem. 259*, 11176–11179.
Fries, H. H. (1886) *Ber. Dtsch. Chem. Ges. 19*, 242–244.
Gahmberg, C. G., & Andersson, L. C. (1977) *J. Biol. Chem. 252*, 5888–5894.
Gan, J., Papkoff, H., & Li, C. H. (1968) *Biochim. Biophys. Acta 170*, 189–197.
Geoghegan, K. F., Dallas, J. L., & Feeney, R. E. (1980) *J. Biol. Chem. 255*, 11429–11434.
Giotta, G. J. (1976) *J. Biol. Chem. 251*, 1247–1252.
Hall, C., & Ruoho, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4529–4533.
Haugland, R. P., Ed. (1981) *Handbook of Fluorescent Probes*, Molecular Probes Inc., Junction City, OR.
Jesaitis, A. J., & Fortes, P. A. G. (1980) *J. Biol. Chem. 255*, 459–467.
Jørgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.
Jørgensen, P. L. (1982) *Biochim. Biophys. Acta 694*, 27–68.
Kaiser, D. W., Thurston, J. T., Dudley, J. R., Schaefer, F. C., Hechenbleikner, I., & Holm-Hansen, D. (1951) *J. Am. Chem. Soc. 73*, 2984–2986.
Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111–136.

Knowles, J. R. (1965) *Biochem. J. 95*, 180-190.
Koepsell, H. (1979) *J. Membr. Biol. 48*, 69-94.
Kyte, J. (1975) *J. Biol. Chem. 250*, 7443-7449.
Kyte, J. (1981) *Nature (London) 292*, 201-204.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Lee, J. A., & Fortes, P. A. G. (1982) *Biophys. J. 37*, 149a.
Lee, J. A., & Fortes, P. A. G. (1983) *Biophys. J. 41*, 369a.
Lo, T. N., & Titus, E. O. (1978) *J. Biol. Chem. 253*, 4432-4438.
Markwell, M. A., Hans, S. M., Bieber, L. L., & Talbert, N. E. (1978) *Anal. Biochem. 193*, 206-210.
Moczydlowski, E. G., & Fortes, P. A. G. (1980) *Biochemistry 19*, 969-977.
Moczydlowski, E. G., & Fortes, P. A. G. (1981a) *J. Biol. Chem.* 256, 2346-2356.
Moczydlowski, E. G., & Fortes, P. A. G. (1981b) *J. Biol. Chem. 256*, 2357-2366.
Mosher, H. S., & Whitmore, F. C. (1945) *J. Am. Chem. Soc. 67*, 662-664.
Reines, S. A., & Cantor, C. R. (1974) *Nucleic Acids Res. 1*, 767-786.
Sabatini, D., Golman, D., Sabban, E., Sherman, J., Morimoto, T., Kreibich, G., & Adesnik, M. (1981) *Cold Spring Harbor Symp. Quant. Biol. 46*, 807-818.
Sadtler Standard Ultraviolet Spectra, Sadtler Research Laboratories, Inc., Philadelphia, PA.
Segrest, J. P., & Jackson, R. L. (1972) *Methods Enzymol. 28*, 54-63.
Shaper, J. H., & Stryer, L. (1977) *J. Supramol. Struct. 6*, 291-299.
Stewart, W. W. (1981) *J. Am. Chem. Soc. 103*, 7615-7620.
Thurston, J. T., Dudley, J. R., Kaiser, D. W., Hechenbleikner, I., Schaefer, C., & Holm-Hansen, D. (1951) *J. Am. Chem. Soc. 73*, 2981-2983.
Tressel, P., & Kosman, D. J. (1980a) *Anal. Biochem. 105*, 150-153.
Tressel, P., & Kosman, D. J. (1980b) *Biochem. Biophys. Res. Commun. 92*, 781-786.
Zantema, A., Maassen, J. A., Kriek, J., & Möller, W. (1982) *Biochemistry 21*, 3069-3076.

Use of Secondary Isotope Effects and Varying pH To Investigate the Mode of Binding of Inhibitory Amino Aldehydes by Leucine Aminopeptidase†

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ABSTRACT: K_i values for leucine aldehyde, a competitive inhibitor of leucine aminopeptidase, vary with pH in a manner compatible with binding of uncharged inhibitor. The pH dependence of k_{cat}/K_m suggests likewise that the substrate leucine *p*-nitroanilide is productively bound as the uncharged species. Comparison of pK_a values of the model compounds aminoacetone and aminoacetal indicates that the equilibrium constant for hydration of amino aldehydes is reduced by a factor of about 2 when a proton is lost from the α-ammonium group near pH 8. Effects of deuterium substitution at C-1 on equilibrium binding of leucine aldehyde were determined with immobilized enzyme and inhibitors doubly labeled with radioisotopes. The observed isotope effect (K_D/K_H) is approximately unity, suggesting that leucine aldehyde combines with the enzyme as an oxygen adduct, not as the intact aldehyde.

The principle of transition-state analogy has been helpful in designing reversible enzyme inhibitors that much surpass substrate analogues in their binding affinities. In cases where an inhibitor of this kind could be bound in any of several possible forms, the actual mode of binding that is observed can be helpful in attempting to distinguish between possible mechanisms (Wolfenden, 1980). Simple amino aldehydes (Andersson et al., 1982), and also amino acid hydroxamates (Chan et al., 1982; Coletti-Previero et al., 1982; Wilkes & Prescott, 1983), have recently been found to serve as strong inhibitors of leucine aminopeptidases from kidney. Amino aldehydes also prolong the analgesic effects of enkephalins and related peptides in vivo, perhaps by preventing their degradation (Davis et al., 1983). This paper describes experiments designed to secure structural information about the binding of amino aldehydes by cytosolic leucine aminopeptidase.

In aqueous solution, α-amino aldehyde cations exist as minor (ca. 2.5%) constituents of an equilibrium mixture consisting mainly of their covalent hydrates or *gem*-diols (Andersson et al., 1982). Influences of pH on inhibition, and on productive binding of substrates, were examined first. The effect of pH on covalent hydration of amino aldehydes (and the corollary influence of hydration on ionization) was then estimated by determining the pK_a values of a model ketone and acetal.

To distinguish between modes of binding, it seemed possible to exploit the known effect of deuterium substitution in the aldehyde group in promoting equilibrium addition of nucleophiles (Lewis & Wolfenden, 1977a). If an inhibitory aldehyde were taken up by the enzyme intact, then its binding would be expected to reflect, almost in full, the 1.37-fold difference in equilibria of hydration between aldehydes and 1-^{2}H-labeled

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