Photoaffinity Labeling of (Na⁺K⁺)-ATPase with [¹²⁵I]Iodoazidocymarin*

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A radioiodinated, photoactive cardiac glycoside derivative, 4'-(3-iodo-4-azidobenzene sulfonyl)cyarin (IAC) was synthesized and used to label (Na⁺K⁺)-ATPase in crude membrane fractions. In the dark, IAC inhibited the activity of (Na⁺K⁺)-ATPase in electrophorax microsomes from Electrophorus electricus with the same Iₐ₀ as cyarin. [¹²⁵I]IAC binding, in the presence of Mg²⁺ and P_i, was specific, of high affinity (K_d = 0.4 μM), and reversible (k₋₁ = 0.11 min⁻¹) at 30 °C. At 0 °C, the complex was stable for at least 3 h, thus permitting washing before photolysis. Analysis of [¹²⁵I]IAC photolabeled electrophorax microsomes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7-14%) showed that most of the incorporated radioactivity was associated with the γ (M_r = 98,000) and β (M_r = 44,000) subunits of the (Na⁺K⁺)-ATPase (ratio of α to β labeling = 2.5). A higher molecular weight peptide (100,000), similar in molecular weight to the brain α(+), subunit, and two lower molecular weight peptides (12,000-15,000), which may be proteolipid, were also labeled. Two-dimensional gel electrophoresis (isoelectric focusing then SDS-PAGE, 10%) resolved the β subunit into 12 labeled peptides ranging in pI from 4.3 to 5.5. When (Na⁺K⁺)-ATPase in synaptosomes from monkey brain cortex was photolabeled and analyzed by SDS-PAGE (7-14%), specific labeling of the α (+), α, and β subunits could be detected (ratio of α(+) plus α to β labeling = 35). The results show that [¹²⁵I]IAC is a sensitive probe of the cardiac glycoside binding site of (Na⁺K⁺)-ATPase and can be used to detect the presence of the α(+) subunit in crude membrane fractions from various sources.

Sodium and potassium ion gradients are maintained across the plasma membrane of most animal cells by the enzyme (Na⁺K⁺)-ATPase. This enzyme maintains the gradient by an ATP-dependent process in which sodium is transported out of the cell and potassium is transported into the cell. The enzyme is specifically inhibited by cardiotonic steroids such as ouabain and cymarin. (Na⁺K⁺)-ATPase, purified from several sources, consists of at least two polypeptides: an α subunit (M_r = 100,000) and a β carbohydrate-containing subunit (M_r = 44,000) (1, 2). The α subunit contains the ATP-phosphorylation site and the cardiac glycoside binding site. The function of the β subunit is presently unknown. Small peptides (M_r = 10,000-12,000), referred to as proteolipid or γ subunits, have been identified but the nature and function of these peptides is also unknown.

Tritium-labeled photoactive derivatives of cardiotonic steroids have been used to specifically label the ouabain binding site on (Na⁺K⁺)-ATPase. Specific labeling of the α subunit occurred when the photoactive moiety of the photoaffinity label was on the C-3 of digitoxigenin (3), the C-17 of digitoxin (4), the C-19 of amide benzothin (5), the rhaminose of ouabain (6, 7), or on the digoxose of digoxin monoglycoside (7). These results are consistent with the ouabain binding site being located primarily on the α subunit of the enzyme. However, specific labeling of the β subunit as well as the α subunit was observed when lubrol-purified electric eel enzyme was labeled with a probe which had the photoactive moiety farther away from the A ring of the steroid (i.e. 4'-diazaizinol-bisdigoxiside or 4'-diazaizinol-digoxitin (7). Thus, the β subunit may also contribute to the structure of the binding site, perhaps in the “sugar specific” region (8). A better understanding of the structure of the binding site and in particular the interface between the α and β subunits in the site may explain how the subunits interact and suggest possible functions for the β subunit.

Two major difficulties which limit investigations of the native structure of the cardiotonic steroid binding site are: (i) the cardiotonic steroid photoaffinity labels that are presently available all contain tritium in the aglycon portion of the molecule with the photoactive group attached separately through an ester or amide bond so that radioactivity is not lost from the enzyme during amino acid and peptide analysis. They must also have sufficiently high specific activity to detect the photoaffinity labeled enzyme in crude membrane preparations (i.e. in situ). The native structure of the enzyme should be investigated in situ without the potential complications resulting from the use of detergents.

To address the question of topographical arrangement of α and β subunits around the ouabain binding site, particularly in preparations of native, membrane-bound (Na⁺K⁺)-ATPase, new photoaffinity labels are needed. These new photo-labels must have the radioactive and photoactive groups on the same side of potentially hydrolyzable bonds so that radioactivity is not lost from the protein during amino acid and peptide analysis. They must also have sufficiently high specific activity to detect the photolabeled enzyme in crude membrane preparations (i.e. in situ).

In this paper we report the synthesis, characterization, and application of [¹²⁵I]IAC (Fig. 1) as a probe for the cardiac glycoside binding site of (Na⁺K⁺)-ATPase in microsomes.

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1 The abbreviations used are: IAC, iodoazidocymarin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NP40, Nonidet P-40.
from electric eel electroplax and in synaptosomes from monkey brain cortex. In electroplax microsomes and in brain synaptosomes, \[^{125}\textrm{I}]\text{IAC} was found to specifically label all of the peptides (\(\alpha(+)\), \(\alpha\), \(\beta\), and \(\gamma\) subunits) which have previously been demonstrated by various methods to be associated with (Na\(^{+}\)K\(^{+}\))-ATPase.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

The data for the chemical characterization of IAC, the inhibition of (Na\(^{+}\)K\(^{+}\))-ATPase activity and binding of \[^{125}\textrm{I}]\text{IAC} to the enzyme are presented in the Miniprint.

**Rationale for the Preparation and Use of IAC**—The orthoiodoazidophenyl group used in this derivatization has several advantages over photoactive groups used successfully in the past, i.e. the diazomalonyl group (3) and the meta-nitrozidophenyl group (5, 6). (i) The ortho-iodoazidophenyl group can be synthesized with \(^{125}\textrm{I}\) at a theoretical specific activity of 2200 Ci/mmol. (ii) After photolysis of the azide and subsequent covalent insertion of the nitrene into the binding site, the radioactivity will remain with the peptide even if the sulfonyl ester bond were to be hydrolyzed since the radioactive atom is part of the photoactive moiety rather than a part of the aglycon. (iii) The use of \(^{125}\textrm{I}\) also has the advantage that, since its emission energy is very high, direct and facile autoradiography of photolabeled material in electrophoretic gels can be performed.

We used cymarin as the parent cardiotonic glycoside because it has a single hydroxyl group on its cymarose residue which can be derivatized. Acetylation of cymarin at the 4' position of the sugar residue is known not to affect the dissociation rate constant of the glycoside (8) and derivatization at the same position with a diazomalonyl group has been shown not to affect cymarin’s ability to inhibit (Na\(^{+}\)K\(^{+}\))-ATPase (20). The results presented in the Miniprint show that modification of cymarin at the 4' position with 3-iodo-4-azidobenzene sulfonyl chloride did not interfere with either the inhibitory activity of cymarin or the high affinity binding (Figs. 2 and 3).

**Photoaffinity Labeling of (Na\(^{+}\)K\(^{+}\))-ATPase in Eel Electroplax Microsomal Membrane**—The concentration dependence of \[^{125}\textrm{I}]\text{IAC} photoaffinity labeling in the eel electroplax microsomal membrane is shown in Fig. 6A. Five radiolabeled bands were resolved by SDS-PAGE analysis of the photolyzed (Na\(^{+}\)K\(^{+}\))-ATPase. The catalytic \(\alpha\) subunit (band a, Fig. 6A) and the glycoprotein \(\beta\) subunit (band c, Fig. 6A), which were identified by Coomassie staining of the gel, were heavily labeled. In addition, a band of radioactivity (band d, Fig. 6A) was detected which had a molecular weight approximately 2000 greater than the \(\alpha\) subunit. This polypeptide has a molecular weight which is similar to the \(\alpha(+)\) subunit form of (Na\(^{+}\)K\(^{+}\))-ATPase first observed in brine shrimp larvae (21) and in brain (18). A corresponding Coomassie stained band for the \(\alpha(+)\) band was not visible, indicating that there was little \(\alpha(+)\) subunit relative to the \(\alpha\) subunit of (Na\(^{+}\)K\(^{+}\))-ATPase in eel electroplax. The presence of the \(\alpha(+)\) subunit has not been previously identified in the electric eel enzyme.

Two specifically radiolabeled peptides of low molecular weight were also identified (bands d and e, Fig. 6A). Based on the molecular weights, one or both of these peptides may be proteolipid components similar to those previously reported to be associated with (Na\(^{+}\)K\(^{+}\))-ATPase (5, 6). The two peptides are referred to here as P-1 and P-2; they have, respectively, approximate molecular weights of 15,000 and 12,000.

Eel electroplax microsomes preincubated with \[^{125}\textrm{I}]\text{IAC} could be washed by dilution and sedimentation prior to photolysis since the dissociation rate of the enzyme-IAC complex could be washed by dilution and sedimentation prior to photolysis.
at 0 °C was very slow (Fig. 3). Fig. 6B shows that washed microsomes were essentially free of nonspecific radiolabeling although Coomassie staining of the gel shows that there were a great number of peptides in these preparations (Fig. 6C). Washing did not affect the specific radiolabeling of the α(+), α, β, P-1, and P-2 bands indicating that these peptides are associated with the cardiotonic steroid binding site.

The total photoincorporation of [125I]IAC at a concentration of 0.16 nM into the five specifically labeled bands was calculated in a representative photolabeling experiment (gel not shown) to be 3.8 pmol of [125I]IAC/mg of protein. Since bound and free [125I]IAC were at equilibrium at the time of photolysis, the number of occupied sites was calculated based on the equilibrium binding presented in Fig. 3 and was 84.4 pmol/mg of protein. From these two values, the efficiency of specific photoincorporation was estimated as 4.5% for the photolysis conditions described in Fig. 6.

Structure-activity studies (8, 22) on the inhibition of (Na,K+)-ATPase by cardiotonic steroids have suggested that there is a region in the binding site of the enzyme which can interact specifically with the sugar moiety of a cardiotonic steroid. It is likely, then, that the photolabels which have their photoactive group located on the sugar moiety are covalently linked after photolysis to this sugar specific region of the binding site. In photolabeling experiments on eel electrophoresis (Na,K+)-ATPase with nitroazidophenyl-ouabain, Rogers and Lazdunski (5) showed specific radiolabeling of the α subunit of the enzyme and proteolipid (Mr = 12,000). Similar observations of specifically photolabeled proteolipid from pig and lamb kidney enzyme using nitroazidobenzoyl-ouabain were reported by Forbush et al. (6). In these studies it was concluded that there was a small protein associated with the sugar specific binding site. On the other hand, Hall and Ruoho (19) showed ouabain protectable photolabeling of both the α and β subunits of the eel electrophoresis enzyme by no detectable labeling of the proteolipid with 4'-diazomalonyl-digitoxin. They concluded that the two major subunits of the enzyme are in intimate contact in the region of the sugar specific binding site. The specific radiolabeling of α, β, and P-2, with [125I]IAC is consistent with both of the conclusions described above for (Na,K+)-ATPase in eel electrophoresis microsomes.

The differences observed between 4'-diazomalonyl-digitoxin, nitroazidophenyl-ouabain, and IAC in the photolabeling of peptides from eel electrophoresis (Na,K+)-ATPase may be related to the type of photoreactive group used and the manner in which the group is attached to the cardiac glycoside. Specific [125I]IAC incorporation into both P-2 and the β subunit in addition to the α subunit may occur for at least two reasons: (i) IAC possesses a hydrophobic photoreactive group which can partition into proteolipid and interact with P-2. The photoreactive groups of IAC, nitroazidobenzoyl-ouabain, and nitroazidophenyl-ouabain are hydrophobic phenyl azides and may partition into proteolipid near the binding site. On the other hand, the photoreactive group of 4'-diazomalonyl-digitoxin is less hydrophobic than the phenyl azide and may not partition as readily into hydrophobic regions. (ii) The photoreactive group of IAC may be positioned favorably for reaction with the β subunit of the enzyme because the phenyl azide moiety in IAC is attached to cymarose which can hydrogen bond to the sugar specific region of the cardiac glycoside binding site of the enzyme. Studies on the dissociation rates of various cardiac monoglycosides from (Na,K+)-ATPase (8) have indicated that the 3'-α-hydroxyl or the 3'-α-methoxyl groups of the sugar moiety are important in stabilizing the ligand-enzyme complex, presumably through hydrogen bonding to the enzyme surface. The 3'-α-hydroxyl group of ouabain is not present in either nitroazidophenyl-ouabain or nitroazidobenzoyl-ouabain and the β subunit is not labeled with these compounds. On the other hand, the 3'-α-methoxyl group of cymarain and the 3'-α-hydroxyl group of digitoxin are intact in IAC and 4'-diazomalonyl-digitoxin, respectively, and they do photolabel the β subunit of (Na,K+)-ATPase.

Two-dimensional Electrophoresis of Labeled Eel Electroplax—Eel electrophoresis microsomal membrane was photolabeled with [125I]IAC as described in Fig. 6B and then analyzed by two-dimensional gel electrophoresis with isoelectric focusing in the first dimension. The results are shown in Fig. 7 and indicate that the radiolabeled band corresponding to the β subunit on SDS-PAGE gels was composed of approximately 12 individual peptides with pI values ranging from 4.3 to 5.5. There was no detectable change in the pI values of the peptides upon specific covalent modification with [125I]IAC since the radioactive spots (Fig. 7B) corresponded precisely with the Coomassie staining spots (Fig. 7A) in the molecular weight range of 44,000. This microheterogeneity of the β subunit agrees with that reported by Marshall and Hokin (23); they had evidence that this heterogeneity is due to variations in the sialic acid content of the subunit.

Some of the protein from the electrophoresis membrane remained in the stacking gel of the first dimension as indicated by the streak of Coomassie-stained protein on the right side of the SDS-polyacrylamide slab gel shown in Fig. 7A. This protein, which was specifically radiolabeled (Fig. 7B), probably contained the α subunit since only a small portion of the radiolabeled α subunit which was loaded onto the isoelectric focusing gel was resolved in both dimensions. Radiolabeled areas above the α subunit shown in Fig. 7B may consist of various combinations of α and β subunits which remained undissociated.

IAC Photoaffinity Labeling of Brain (Na,K+)-ATPase—Iodinated photolabels are better suited for identifying low density binding sites in crude membrane preparations than are tritiated photolabels since [125I]IAC has high specific activity and emission energy (7). In order to test whether (Na,K+)-ATPase can be radiolabeled sufficiently for visualization in tissues where the amount of the enzyme relative to the total protein is lower than in eel electrophoresis, crude synaptosomes from monkey brain were photolabeled with [125I]IAC. The specific (Na,K+)-ATPase activity in crude monkey brain

![Fig. 7. Two-dimensional electrophoresis of [125I]IAC photoaffinity labeled eel electrophoresis microsomal membrane. A washed sample, prepared as described in Fig. 6B (no ouabain in preincubation), was analyzed by two-dimensional electrophoresis as described under "Experimental Procedures." A, Coomassie protein stain of the two-dimensional gel. The molecular weight standards × 10^3 indicated are: myosin, 220; phosphorylase A, 94; catalase, 60; actin, 43; and lysozyme, 14 (Kendrick Laboratories, Madison, WI). The arrow indicates the isoelectric focusing standard vitamin D-dependent calcium binding protein (Mr = 27,000), apparent pI 4.2; Kendrick Laboratories. The pH gradient of the first dimension (isolectric focusing) is indicated at the bottom of the autoradiogram. B, Two-month autoradiogram of the two-dimensional gel.](image-url)
synaptosomes was less than 5% of that found in eel electroplax microsomes. Analysis of photolabeled brain synaptosomes by SDS-PAGE (Fig. 8, C–E) showed a radiolabeled peptide doublet (difference in $M_r = 2,000$) in the molecular weight range of the $\alpha$ subunit of eel electroplax (Na$^+$K$^+$)-ATPase (Fig. 8, A–B). The labeling of both peptides was found to be very specific as indicated by ouabain protection and by the lack of label in other membrane proteins (compare the Coomassie profile in Fig. 8E with the photolabeling pattern in Fig. 8D). Thus, $[^{125}]$IAC photolabeling of monkey brain cortex suggests that two forms of the catalytic subunit of (Na$^+$K$^+$)-ATPase, $\alpha(\text{+})$ and $\alpha$, are present in primate brain. Sweadner (18) identified these two forms in brain from calf, dog, frog, mouse, and rat. She also found that rat brain axolemma contained the $\alpha(\text{+})$ form and rat brain astrocytes contained the $\alpha$ form. This suggests that the ratio of radiolabeled $\alpha(\text{+})$ to radiolabeled $\alpha$ for photolabeled brain (Na$^+$K$^+$)-ATPase shown in Fig. 8D is probably related to the ratio of axolemma membrane to astrocyte membrane in the synaptosome preparation.

A 3-week autoradiogram of the same gel used in Fig. 8D revealed specific labeling of a diffuse area (position c, Fig. 8C) with the same molecular weight as the $\beta$ subunit of eel electroplax (Na$^+$K$^+$)-ATPase (Fig. 8, A–B). Some specific labeling of low molecular peptides, P-1 and P-2, could be detected (positions d and e, Fig. 8C). Some other radiolabeled peptides which were not ouabain protectable can be seen after this long exposure time. The efficiency of insertion into the glycoprotein subunit, relative to insertion into the catalytic ($\alpha(\text{+})$ plus $\alpha$) subunits, was not the same for eel electroplax microsomes as compared with monkey brain synaptosomes. The ratio of specific labeling of the catalytic subunits to the $\beta$ subunit, determined from $\gamma$-counting of gel slices, was found to be 35 to 1 for monkey brain enzyme whereas the same ratio was found to be 2.4 to 1 for eel electroplax enzyme. Thus, photoinertion into the $\beta$ subunit of the enzyme appears to be a function of both the type of cardiac glycoside photolabel used and the type of (Na$^+$K$^+$)-ATPase labeled.

In summary, a comparison of photolabeled crude membranes from eel electroplax and monkey brain indicates that the (Na$^+$K$^+$)-ATPase in these tissues appear to have the same binding-site specific peptides. A difference between the two sources of enzyme is in the efficiency of radiolabeling of the minor peptides ($\beta$ subunit, P-1, P-2) relative to the $\alpha$ subunit labeling. These results suggest that the topographical arrangement of the peptides in the native conformation of the enzyme may vary with the tissue source. The detection of an $\alpha(\text{+})$ form of the catalytic subunit in electroplax as well as in brain suggests that both forms may be present in many tissues. Further comparisons of native enzyme with respect to species and tissue type should be greatly facilitated through the use of $[^{125}]$IAC and similar ortho-iodoazidophenyl cardiotonic steroid derivatives.

REFERENCES
Supplemental Material to
Photolabeling of Na-K-ATPase
with [125I]iodoacetamide

by
Joseph M. Lovendes, Robert Botwin-Narseter and Arnold E. Bush

Experimental Procedures

Materials

Radiolabeled reagents were purchased from New England Nuclear (Boston, MA). [125I]ATP was synthesized in our laboratory by using the method of de Weck and Lebeaux (34). 4-Chloro-2-nitrophenylalanine (St. Louis, MO) was obtained as a gift from Dr. Jack L. Hempel, JR. from the Department of Pharmacology, University of Wisconsin, Madison. Monkey cerebral cortex microsomal membrane fractions were prepared in our laboratory by Dr. David L. Lasek as previously described (12).

Inhibition of Na-K-ATPase. The inhibitory activity of IAC was determined by using the method of Manger (35). 1 mg of purified Na-K-ATPase was prepared in 50 mM HEPES buffer (pH 7.4) containing 100 mg/ml bovine serum albumin. The enzyme preparation was incubated with 10% glycerol and stored at -70°C. The specific activity was determined by removal of inorganic phosphate with the use of a Packard model 3000 liquid scintillation spectrometer. The assay contained 200 ml of 100 mM HEPES buffer (pH 7.4), 100 mM NaCl, 100 mM KCl, 1 mM EDTA, 1 mM ATP, 0.025 mCi [125I]iodoacetamide, and 100 mg/ml bovine serum albumin. The reaction was stopped after 10 min by the addition of 0.5 ml of 10% trichloroacetic acid. The precipitate was washed five times with 100 ml of 10% trichloroacetic acid and dissolved in 1 M NaOH. The samples were measured for radioactivity with a Packard model 3000 liquid scintillation spectrometer.

[125I]IAC Photolabeled (Na+K+)-ATPase

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Fig. 1. Inhibition of Na-K-ATPase activity by cytarabine and lodoxadroxarin under non-equilibrium conditions. Na-K-ATPase activity in the presence of varying concentrations of cytarabine (I) or IAC (II) was determined as described under Methods and expressed as a percentage of the control. The control was specific Na-K-ATPase activity in the absence of cytarabine and IAC (1.5 pmol Pi min⁻¹ mg⁻¹ protein).

Specific [¹²⁵I]IAC binding. [¹²⁵I]IAC bound specifically, and with high affinity, to rat stomach microsomal Na-K-ATPase under equilibrium binding conditions (Fig. 2A). The equilibrium dissociation constant (K_d) was determined in a Scatchard plot analysis to be 0.49 μM (Fig. 2B). The number of binding sites was successfully determined to be 128 pmol/mg protein. The [¹²⁵I]IAC-Na-K-ATPase complex was reversible in the dark (Fig. 2A).

The rate of dissociation of the complex was temperature dependent; dissociation at 20°C was rapid while dissociation at 0°C was too slow to measure over the 3 hour interval studied. The first-order dissociation rate constant (k_d) at 20°C was 0.11 min⁻¹ (Fig. 2B), giving a half-life of the enzyme-photolabeled complex of 6.7 minutes. The halflife derived from the Scatchard plot analysis (6.3 min) agreed well with the observed value.

The data suggested the existence of a second form of Na-K-ATPase with a different K_d. Two forms of Na-K-ATPase with different inhibitor binding properties have been found in the brain (9).

The slow rate of dissociation of the IAC-photolabeled Na-K-ATPase complex at 0°C was used to photolabel Na-K-ATPase with [¹²⁵I]IAC. After incubation with [¹²⁵I]IAC at 20°C, membrane samples were quickly cooled to 0°C and then either diluted or washed by centrifugation before photolysis. This technique was used to reduce the amount of nonspecific [¹²⁵I]IAC binding with a minimal loss of specific [¹²⁵I]IAC binding. Background photolabeling was therefore reduced relative to specific photolabeling.

Fig. 2A: Equilibrium binding of [¹²⁵I]IAC to Na-K-ATPase with electrophoresis microsomal membranes, determined as described under Methods. In the presence (I) or absence (II) of 0.1 μM inhibitor. B: Scatchard plot analysis of the specific binding calculated from the data shown in Fig. 2A.

Fig. 2B: Dissociation rate of the [¹²⁵I]IAC-photolabeled Na-K-ATPase complex, determined as described under Methods, at 20°C (I) and 0°C (II). The first-order dissociation rate constant (k_d) was determined from the slope of the plot of ln(binding/initial binding) versus time. Binding/initial binding is abbreviated B/I.

[¹²⁵I]IAC photolabeling. Samples of Na-K-ATPase-enriched electrophoresis membranes were incubated with [¹²⁵I]IAC and exposed to light for varying lengths of time. For a dark control, a sample was incubated with [¹²⁵I]IAC but not exposed to light. Fig. 3A shows an autoradiograph of the samples after analysis by SDS-PAGE. Specific incorporation of [¹²⁵I]IAC was light dependent, and the autoradiograph showed the expected incorporation of [¹²⁵I]IAC into the Na-K-ATPase while samples exposed to light showed specific labeling of Na-K-ATPase. The rate of photolabeling of the α-subunit appeared to be faster than the rate of photolabeling of the β-subunit. Fig. 3B is a plot of the radioactivity present in gel slices from the 20 second photolysis shown in Fig. 3A. The α-subunit was identified by its molecular weight and location on the gel. Specific labeling of the α-subunit was consistent with results obtained with nitrocellulose dipropionyl diphenyl derivatives (10). Since these membranes were not treated with detergents prior to photolysis, the results indicate that the α- and β-subunits are structurally positioned very close to each other in Na-K-ATPase. In addition, low molecular weight peptides, tentatively identified as the same phosphorylated seen by other laboratories in Na-K-ATPase, were not photolabeled specifically. Specific labeling at the top of the gel tracks was not detected (Fig. 3B) indicating that the photolytic crosslinking of α-subunits (11) did not occur.

Fig. 3A: Light-dependent covalent incorporation of [¹²⁵I]IAC into Na-K-ATPase (a: α-subunit, b: β-subunit) under conditions of light and dark incubation (10). General incubation and photolysis conditions were as described in Methods. Electrophoresis was performed on 0.5 μM protein/membrane. Samples were incubated with 0.1 μM Na-K-ATPase in the presence (I) or absence (II) of 0.1 μM inhibitor. One sample was not photolyzed. The remaining samples were exposed to light for 2, 4, 12, or 20 seconds. The membranes were washed with PBS after exposure to light. Autoradiographs were prepared as described in Methods. The autoradiographs were then developed and exposed to X-ray film for 20 seconds. A: Autoradiograph of the samples after analysis by SDS-PAGE. B: Plot showing the radioactivity present in gel slices from the 20 second photolysis shown in Fig. 3A. The α-subunit was identified by its molecular weight and location on the gel. Specific labeling of the α-subunit was consistent with results obtained with nitrocellulose dipropionyl diphenyl derivatives (10). Since these membranes were not treated with detergents prior to photolysis, the results indicate that the α- and β-subunits are structurally positioned very close to each other in Na-K-ATPase.