Proc. Nat. Acad. Sci. USA
Vol. 71, No. 6, pp. 2352–2356, June 1974

Photoaffinity Labeling of the Ouabain-Binding Site on ($Na^+ + K^+$) Adenosinetriphosphatase

(cardiac glycoside/membrane enzyme/active transport)

ARNOLD RUOHO AND JACK KYTE

Department of Biology, University of California at San Diego, La Jolla, Calif. 92037

Communicated by S. J. Singer, February 27, 1974

ABSTRACT **An ethyl diazomalonyl derivative of cymarin was synthesized in order to photoaffinity label the cardiac glycoside-binding site on ($Na^+ + K^+$) adenosinetriphosphatase (EC 3.6.1.3). When a noncovalent complex of the enzyme and this cardiac glycoside derivative was photolyzed, a covalent bond was formed between the ligand and the larger of the two polypeptide subunits of the enzyme. Several control experiments demonstrate that this photochemical reaction occurred while the ligand was bound to the site at which it inhibits the enzyme activity. Another specific inhibitor, tentatively identified as the ethyl chloromalonyl derivative of cymarin, produced similar photoaffinity labeling of the larger subunit, demonstrating that the photolytic dissociation of the diazo group may not be responsible for the photochemical reaction. Since the cardiac glycoside-binding site, which is accessible from the outside surface of the plasma membrane, and the phosphorylation site, which is accessible from the inside surface, are both on the larger polypeptide subunit of ($Na^+ + K^+$) adenosinetriphosphatase, this polypeptide has sequences exposed to both sides of the membrane.**

($Na^+ + K^+$) adenosinetriphosphatase is the membrane-bound enzyme that transports sodium and potassium in opposite directions across the cell membrane to create the ion gradients that are utilized to perform physiological functions. The cardiac glycosides and aglycones, such as digoxin, ouabain, and strophanthidin, are natural products that have been used therapeutically for centuries in the treatment of heart disease. Their only known biochemical effect is to inhibit specifically ($Na^+ + K^+$) adenosinetriphosphatase and consequently decrease or completely stop active cation flux.

The purified enzyme is a specific complex of two polypeptide chains (1). In order to determine which polypeptide contains the cardiac glycoside-binding site, a radioactive affinity label can be used (2). Haloacetyl derivatives of the cardiac glycosides strophanthidin and hellebrigenin (3, 4) were synthesized for affinity labeling of the cardiac glycoside-binding site of ($Na^+ + K^+$) adenosinetriphosphatase. They did not, however, react covalently and specifically with the binding site (5). One disadvantage of such electrophilic reagents is the requirement for a suitably positioned nucleophile in the active site. On the other hand, a photoaffinity label can be converted by photolysis into an exceedingly reactive intermediate, and under appropriate circumstances even insertion into a C–H bond is possible (6). Compounds that generate carbenes or nitrenes upon photolysis have been used for the photoaffinity labeling of enzyme active sites (7), antibody ligand sites (8), and membrane-bound adenosine 3′:5′-cyclic monophosphate binding sites (9). We have synthesized and used an ethyl diazomalonyl derivative of the cardiac glycoside, cymarin (DAMN cymarin, Fig. 1) for photoaffinity labeling of this specific binding site in ($Na^+ + K^+$) adenosine triphosphatase.

Abbreviations: DAMN cymarin, 4′-(ethyl diazomalonyl) cymarin; CM cymarin, 4′-(ethyl chloromalonyl) cymarin; AMP-PNP, adenylyl imidodiphosphate.

MATERIALS AND METHODS

Synthesis of photolabels

Method A. Acylation of cymarin at the 4′-hydroxyl of the cymarose was performed with redistilled ethyl diazomalonyl chloride (10) by a modification of the method of Jacobs (11). A 10-fold molar excess of ethyl diazomalonyl chloride was added to a solution of cymarin in pyridine and the reaction was allowed to proceed for 1 hr at room temperature. The yield of the acylated product was quantitative, and it was recrystallized from methanol–ether. Characterization was performed by infrared and nuclear magnetic resonance spectroscopy.

Method B. In order to perform the reaction on the small scale required for the synthesis of the radioactive derivative, an alternate method of preparing DAMN cymarin was also used. Ethyl diazomalonyl chloride was synthesized from a 3-fold molar excess of ethyl diazoacetate to phosgene, as described by Vaughan and Westheimer (12). One-fifth molar equivalent of cymarin in pyridine was then added directly to this mixture. The acylation was performed *in situ* overnight at room temperature. The products, separated on silica gel G thin-layer plates in 1:1 benzene–acetonitrile, were approximately 80% DAMN cymarin (R_F = 0.45) and 20% of another cymarin derivative (R_F = 0.57). The latter compound has been tentatively identified as 4′-(ethyl chloromalonyl) cymarin (CM cymarin)*.

* This tentative characterization is based on the following observations: (*a*) the 220-MHz nuclear magnetic resonance spectrum of CM cymarin is identical to that of DAMN cymarin except for an additional singlet (1 proton) at δ = 4.89; (*b*) the largest ion in its mass spectrum is at m/e = 678, the molecular weight of CM cymarin–H_2O; (*c*) both nuclear magnetic resonance and mass spectra show that the strophanthidin core is present in an unaltered state; (*d*) there is no diazo absorption band at 2100 cm^{-1} in the infrared spectrum; otherwise CM cymarin and DAMN cymarin display very similar infrared spectra; (*e*) it is radioactive when the synthesis is carried out with [^{14}C]phosgene; (*f*) CM cymarin gives a positive Beilstein test; and (*g*) upon saponification, the radioactivity is released from [^{14}C]CM cymarin as [^{14}C]chloromalonic acid (determined by thin-layer chromatography on polyamide sheets).

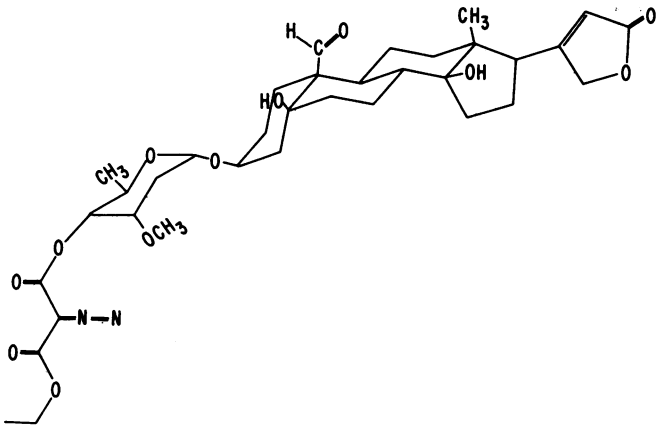

FIG. 1. Structure of DAMN cymarin.

[^{14}C]DAMN cymarin and [^{14}C]CM cymarin were synthesized from [^{14}C]phosgene (5 mCi/mmole) by this latter method (Method B). Purification of the radioactive compounds was achieved on silica gel G thick-layer plates in 1:1 benzene–acetonitrile. The radioactive compounds chromatographed with the pure non-radioactive derivatives in four separate solvent systems on silica gel G thin-layer plates.

Both compounds were reversible inhibitors of ($Na^+ + K^+$) adenosine triphosphatase when assayed in dim light. The K_Is were 1.3×10^{-6} M for DAMN cymarin and 0.9×10^{-6} M for CM cymarin.

Photolysis

A microsomal preparation (extracted by salt detergent) of ($Na^+ + K^+$) adenosine triphosphatase from canine renal medulla was used for these experiments (13). Pyruvate kinase and cymarin were purchased from Sigma, and adenylyl imidodiphosphate (AMPPNP) from ICN.

In order to perform the photolysis experiments, the radioactive photoaffinity label (in 50% benzene–ethanol) was dried down in a cuvette of 2-mm path length; it was dissolved in 20 μl of dimethyl sulfoxide, and then enzyme and buffer were added, so that the final concentrations were 50 mM phosphoenolpyruvate, 150 mM Na^+, 0.5 mM ATP, 1.5 mM EDTA, 7 mM 2-mercaptoethanol, 2.5 mM $MgCl_2$, 0.2 mg/ml of pyruvate kinase, and 30 mM imidazolium·Cl, pH 7.0; in a final volume of 0.1 ml. The mixture was flushed with N_2 for 5 min and photolyzed at 0° under N_2, 1 cm from the filament of a H85A medium pressure Hg lamp. The solution was diluted to 2.0 ml with 1 mM cymarin, incubated at 37° for 15 min, and then centrifuged at 100,000 × g for 20 min. The pellets were dissolved in 0.1 ml 4% sodium dodecyl sulfate, 1% 2-mercaptoethanol at 100° and subjected to electrophoresis on ethylene diacrylate-crosslinked, 5.7% polyacrylamide gels (14). The gels were scanned at 280 nm, and sliced into 2-mm disks. The ester-crosslinked gel slices were dissolved in 15 ml of toluene NCS fluor at 37° overnight. A basic medium was used to dissolve the gel in order to trap any liberated CO_2. No peroxide was used to dissolve the gel to avoid decarboxylation. The dissolved slices were counted in a Beckmann liquid scintillation counter.

RESULTS

Photoaffinity Labeling of ($Na^+ + K^+$) Adenosine Triphosphatase with DAMN Cymarin. Cardiac glycosides bind most tightly to ($Na^+ + K^+$) adenosine triphosphatase when it is in the phosphorylated form (15), which requires the presence of Na^+ and MgATP (16) or Mg^{2+} and P_i (17). Since there is a low level of contaminating, nonspecific adenosine triphosphatase present in the enzyme preparation (13), it was necessary to add an ATP-generating system (namely, phosphoenolpyruvate and pyruvate kinase) when Na^+ and ATP were used to phosphorylate the enzyme. This avoided using a high concentration of MgATP, which would absorb the light used to photolyze the enzyme inhibitor complex. In the presence of Na^+, MgATP, and the ATP-generating system [^{14}C]DAMN cymarin (2×10^{-9} moles, 23,000 cpm) and ($Na^+ + K^+$) adenosine triphosphatase [0.5 mg, 2×10^{-9} moles of cardiac glycoside-binding sites (18)] were mixed together in a 2-mm path length quartz cuvette in a final volume of 0.1 ml. Under these circumstances, both enzyme and inhibitor are present at concentrations in excess of the dissociation constant for the enzyme–inhibitor complex and most of the inhibitor is bound to sites on the enzyme (8). After a short preincubation under nitrogen, the solution was exposed to strong ultraviolet light for 5 min. The reaction product was then analyzed on sodium dodecyl sulfate–polyacrylamide gels (19). The distribution of ^{14}C counts and protein that resulted are shown in Fig. 2. There are three protein components resolved by the electrophoresis; the two polypeptides of ($Na^+ + K^+$) adenosine triphosphatase and a covalently crosslinked dimer of the two chains, which is formed during photolysis (J. Kyte and A. Ruoho, manuscript in preparation). It can be seen that the large chain of ($Na^+ + K^+$) adenosine triphosphatase is radioactively labeled, but not the small chain. Although the number of counts above background was small, in three independent experiments this increment was reproducible.

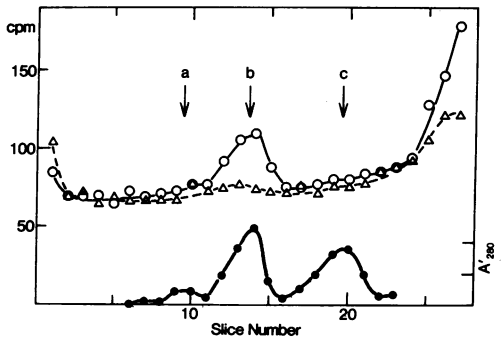

FIG. 2. Distribution of radioactivity covalently attached to ($Na^+ + K^+$) adenosine triphosphatase that has been photolyzed in the presence of [^{14}C]DAMN cymarin. Samples of enzyme that had been photolyzed either in the presence of the complete MgATP phosphorylating system (○) or the complete system plus a 25-fold excess of cymarin as protector (△) were run on sodium dodecyl sulfate gels which were scanned, sliced, and counted. The A_{280} trace from the gel with the unprotected sample was divided into segments exactly as the gel had been sliced. The mean A'_{280} of each of these segments was calculated and the values were plotted (●). The units of A'_{280} are arbitrary since the scanner was uncalibrated. The three protein components are: (*a*) crosslinked $\alpha\beta$ dimer; (*b*) large chain; (*c*) small chain.

In order to demonstrate that this incorporation of radioactivity into the large chain is a result of photoaffinity labeling occurring at the cardiac glycoside site on ($Na^+ + K^+$) adenosine triphosphatase, several controls were performed.

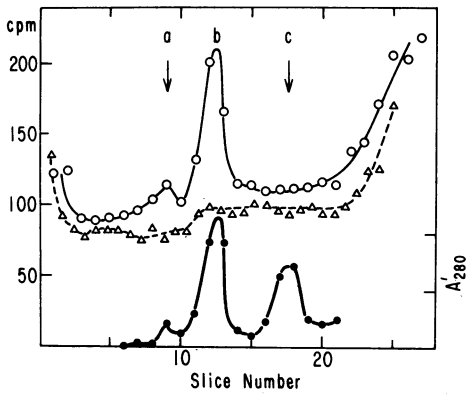

FIG. 3. Distribution of radioactivity covalently attached to $(Na^+ + K^+)$ adenosinetriphosphatase that has been photolyzed in the presence of [^{14}C]CM cymarin. Calculations were performed as described in the *legend* to Fig. 2. Complete MgATP phosphorylating system (○); plus 25-fold excess cymarin (△); A'_{280} (●). (*a*) Covalent $\alpha\beta$ dimer; (*b*) large chain; (*c*) small chain.

(*1*) Presented in Fig. 2 is the distribution of radioactivity for a sample that was photolyzed in the presence of 5×10^{-8} moles of cymarin (25-fold excess) to protect the inhibitor-binding sites. There is no peak of counts on this gel at the position of the large chain of $(Na^+ + K^+)$ adenosine triphosphatase.

(*2*) When the complete reaction mixture was not exposed to light, no radioactivity was covalently incorporated into the protein.

(*3*) When ATP is replaced by its phosphorimidate analog, AMPPNP, the enzyme can no longer phosphorylate and, as expected, no photoaffinity labeling occurs.

On the other hand, labeling of the large chain is observed when the enzyme is phosphorylated in the presence of Mg^{2+} and P_i (17). Table 1 summarizes the results of these experiments. The dark control was used as a measure of background radioactivity in calculating these data.

Photoaffinity Labeling of $(Na^+ + K^+)$ Adenosine Triphosphatase with CM Cymarin. CM cymarin is the product of an unexpected side reaction that occurs when DAMN cymarin is synthesized by Method B. It has a structure very similar to that of DAMN cymarin but lacks the diazo group*. Although the identification of CM cymarin is tentative, the photoaffinity results obtained with it are definitive. It was tested as a photoaffinity label for the cardiac glycoside-binding site and proved to be more efficient than DAMN cymarin. The results of an experiment in which $(Na^+ + K^+)$ adenosine triphosphatase (2×10^{-9} mole sites) and CM cymarin (23,000 cpm; 2×10^{-9} moles) were mixed under phosphorylating conditions and photolyzed for 5 min are displayed in Fig. 3. Three protein components are again resolved, the two polypeptides of the enzyme and the covalent dimer. It can be seen that radioactivity is incorporated into the covalent dimer and the large chain of the enzyme under these circumstances, and within the sensitivity of our measurements, not into the small chain. There are, however, about twice as many cpm incorporated when CM cymarin is used as a photoaffinity label than when DAMN cymarin is used (Tables 1 and 2). When 5×10^{-8} moles of cymarin (25-fold excess) is added to the reaction mixture to protect the cardiac glycoside site on the enzyme, the labeling associated with the large chain of $(Na^+ + K^+)$ adenosine triphosphatase and the covalent dimer of the two chains is eliminated (Fig. 3). The dimer contains one large chain and one small chain, and presumably the radioactivity is attached to the former.

TABLE 1. *Radioactivity covalently attached to the large chain of $(Na^+ + K^+)$ adenosinetriphosphatase that has been photolyzed in the presence of [^{14}C]DAMN cymarin*

Incubation mixture	Total cpm A	B	C	Relative cpm/A_{280}
Complete ATP system	112	108	85	1.00
+ cymarin	19	22		0.20
− ATP + AMPPNP			15	0.10
$Mg^{2+} + P_i$			38	0.97

Enzyme was photolyzed under the conditions noted and run on gels which were scanned, sliced, and counted. The position of the large chain was determined from the scan and the total number of cpm in all the slices from that region was calculated after background was subtracted (determined from the dark control). These data are presented as total cpm. Results from three separate experiments (*A–C*) are tabulated. The total cpm were then divided by the area of the A'_{280} peak of the large chain, calculated from the scan of each gel, and each of these quotients was normalized to that of the complete system to obtain relative cpm/A_{280}.

Several controls were performed to demonstrate that the labeling observed with CM cymarin was occurring at the cardiac glycoside site. They are summarized in Table 2. Again, much less labeling was observed when AMPPNP was substituted for ATP or when photolysis was omitted. The presence of the ATP-generating system is essential to obtain labeling since none occurs when pyruvate kinase is omitted. When Na^+ is replaced by K^+, no labeling is observed. It has been shown that K^+ partially competes with the binding of cardiac glycoside (20).

Specific labeling was observed when the phosphorylated enzyme was formed in the presence of Mg^{2+} and P_i. Although the incorporation of label in the presence of Mg^{2+} and P_i occurs only at the cardiac glycoside site (see controls, Table 2),

TABLE 2. *Radioactivity covalently attached to the large chain of $(Na^+ + K^+)$ adenosinetriphosphatase that has been photolyzed in the presence of [^{14}C]CM cymarin*

Incubation mixture	Total cpm	Relative cpm/A_{280}
Complete ATP system	227 (9)	1.00
+ cymarin	8 (4)	0.06
− Mg^{2+}	11	0.06
− ATP + AMPPNP	59	0.28
− pyruvate kinase	27	0.11
− $Na^+ + K^+$	67	0.12
Dark control	17	0.09
$Mg^{2+} + P_i$	274 (3)	1.80
+ cymarin	65 (2)	0.35
− $Mg^{2+} + Ca^{2+}$	38	0.30
− Mg^{2+}	15 (2)	0.10

The data were calculated as described for Table 1, except that an average of those controls that contained the least amount of radioactivity was used as the measure of the background. The number of times each experiment was performed is in *parentheses*.

the specific activity (cpm/A_{280}) of the large chain labeled under these conditions is twice that of large chain labeled in the presence of Na^+ and ATP. This probably reflects the fact that the absorbance of the former reaction mixture is much lower and more extensive photolysis is possible. There is also, however, an increase in the amount of photolytic crosslinking so that less unaggregated large chain is left in the sample after photolysis. As a result, the total number of cpm attached to large chain is about the same as that observed in the presence of Na^+ and ATP (Table 2). This same problem arose when the time of photolysis was lengthened. When enzyme and CM cymarin were photolyzed in the presence of Na^+ and ATP for 10 min instead of 5 min, the specific activity (cpm/A_{280}) was doubled. Only 20% more cpm, however, were present at the position of the large chain because only 65% as much uncrosslinked large chain remained. For this reason, 5 min was chosen as the optimal photolysis interval.

The radioactivity present at the bottom of the gels when ($Na^+ + K^+$) adenosine triphosphatase is photolyzed in the presence of either DAMN cymarin or CM cymarin (Figs. 2 and 3) is not due to the covalent labeling of any protein component. It is present in the dark control samples, and it can be removed from the gels, before slicing and counting, by soaking them in isopropanol–acetic acid overnight, a procedure that does not remove any of the radioactivity from the large chain or any protein from the gels. This material probably is label that was still noncovalently bound to the enzyme when it was dissolved in sodium dodecyl sulfate.

No peak of radioactivity coinciding with the position of the small chain of ($Na^+ + K^+$) adenosine triphosphatase was observed under any circumstances. Sometimes there were more cpm in this region on the experimental gel than in the control (Fig. 3), but this was not consistently observed. There were never significant quantities of protectable radioactivity in this region nor were the low levels of radioactivity that were present significantly altered in the controls.

For these reasons it is concluded that only the large chain of ($Na^+ + K^+$) adenosine triphosphatase is specifically photoaffinity labeled with these derivatives of cymarin and that it, therefore, contains a portion or all of the cardiac glycoside-binding site.

DISCUSSION

It has been shown that only when cardiac glycoside is present at the outside surface of the cell does it inhibit sodium and potassium transport (21). The enzyme responsible for this active transport process, ($Na^+ + K^+$) adenosine triphosphatase, is a complex of two polypeptides and lipid (1). The larger of the two polypeptides (the large chain) is specifically phosphorylated (22), from intracellular ATP (23), during turnover of the enzyme and has antigenic sites on the inside surface of the membrane (24). When a kinetic titration of the purified enzyme was performed, using a derivative of strophanthidin, it was determined that there was one cardiac glycoside-binding site per large chain (18). This experiment does not specify, however, which polypeptide actually forms the cardiac glycoside-binding site, only that the number of large chains determines the number of sites. The smaller polypeptide (the small chain) is present in the purified enzyme in greater concentration than the large chain, approximately two moles per mole (1, 13). It is a sialoglycoprotein (1) and should have a portion of its surface on the outside of the cell (25). Since both the small chain and the cardiac glycoside-binding site are located on the same side of the membrane, it was conceivable before the present experiments were carried out that the small chain contained that site and the large chain did not directly contribute to it. The results of the experiments described here demonstrate that the large chain of ($Na^+ + K^+$) adenosine triphosphatase contains at least one of the amino-acid seqeunces that compose the cardiac glycoside-binding site on the enzyme. On the other hand, the possibility is not ruled out that the small chain also participates to form the site. The covalent bond-forming event that occurs after the photolysis of the bound reagent may be confined to a very limited region on the enzyme's surface, or the small chain may be labeled but at a level below the limit of detection (<5–10% of the level of incorporation into the large chain)†.

The label attached to the large chain that is observed with either DAMN cymarin or CM cymarin is specific for the cardiac glycoside-binding site. This conclusion follows from the observation that labeling occurs when the enzyme is phosphorylated, does not occur when phosphorylation is prevented, and is not observed when a large molar excess of cymarin is present, which competes for the site (Tables 1 and 2).

It has previously been suggested (28) that the apparent protection against photoaffinity labeling that is afforded by an excess of a protector may sometimes be an artifact due to light absorption by the protector. The protection against the labeling that occurs in the presence of excess cymarin, however, is not due to light absorption artifacts. Complete protection is demonstrated under other conditions in which the absorbance does not change (e.g., minus Mg^{++}, Table 2) or is reduced (e.g., minus pyruvate kinase, Table 2).

It has also been observed that, with some reagents, long-lived, photogenerated radicals can be formed which can then label the protein (28). Under these circumstances, nonspecific labeling is a major problem. The experiments described in this paper were performed in the presence of several potential "scavengers" (mercaptoethanol, imidazole, pyruvate kinase, etc.). Such "scavengers" can react with those excited species of the photolabel formed in solution rather than on the enzyme and aid in reducing this source of nonspecific labeling. To further minimize nonspecific labeling, the concentration of glycoside-binding sites and the concentration of photolabel were equimolar, and the absolute concentration of sites and photolabel was very much greater than the measured K_Is. Under these circumstances, the amount of free steroid in the solution is low. In fact, increasing the concentration of DAMN cymarin by a factor of five resulted in only two times more covalent labeling of sites while the background increased significantly (data not shown).

At this point it is not possible to assess the mechanism by which covalent-bond formation occurs upon photolysis. Both compounds described in this paper are *a posteriori* photolabels. CM cymarin, however, lacks the diazo group, and cannot generate a carbene by the anticipated N_2 dissociation mecha-

† Although both the heavy and light chains of IgG immunoglobulin together form the binding site, it has been observed that the ratio of covalent incorporation of an affinity label into the two chains of different anti-Dnp immunoglobulins varies from 0.1 to 10 (heavy/light) (26). Where two polypeptides contribute to an active site, the ratio of affinity label attached to residues in the two chains can vary over a wide range as a result of relatively small differences in the free energy of activation for reaction with groups on the two chains (27).

nism. This raises the possibility that, with DAMN cymarin as well as with CM cymarin, photolysis generates a reactive species in the glycoside itself, which proceeds to insert into some active site residue(s) in the protein‡. It is clear, however, that the ouabain-binding site of the $(Na^+ + K^+)$ adenosine triphosphatase is being specifically labeled as the result of a photolytic event because radioactivity is incorporated into the large chain only in response to light.

The radioactivity covalently incorporated into the enzyme is low (less than 2% of the total ^{14}C added during photolysis). As mentioned previously, it is possible that the photolytic event is occurring at functional groups other than the diazo group. The efficiency with which this event might occur is not known. If the molar concentration of water in the region of the diazo group is very high, then insertion of a generated carbene will occur preferentially into water and not into the protein§. Reaction of 80% of a carbene with water rather than the enzyme has been observed by Westheimer and his colleagues in the photolysis of diazoacetyl chymotrypsin (29) and, more recently, in the diazoacetyl subtilisin (7). Finally, it was not possible to photolyze the enzyme–inhibitor complex in these experiments for extended periods of time due to the competing reaction of photolytic crosslinking. For this reason, we do not know how much more label would be inserted if the photoreaction were allowed to go to completion¶.

Since the cardiac glycosides bind to the enzyme only at the outside surface of the cell, a portion of the amino-acid sequence of the large chain of the enzyme must be located on the outside surface of the cell. Antibodies have been produced against purified large chain of $(Na^+ + K^+)$ adenosine triphosphatase, and the antigenic sites to which these are directed are located on the inside surface of the cell (24). It is also known that the large chain is phosphorylated (22) by MgATP from the inside of the cell (23). Consequently, the large chain must have a surface exposed to the outside of the cell and one exposed to the inside and, therefore, probably spans the membrane. In the absence of the information contained in this paper, that the large chain contributes directly to the cardiac glycoside site, this statement could not be made. The molecular weight of the large chain of $(Na^+ + K^+)$ adenosine triphosphatase is 130,000, and it has been pointed out (1) that if it were folded as a compact sphere its diameter would be 75 Å, more than enough to span the membrane with adequate surfaces on each side.

We thank S. J. Singer for his advice and support. We also thank Dr. Charles Perrin for helpful discussions. A.R. is a Helen Hay Whitney Postdoctoral Fellow; J.K., a Damon Runyon Fund Postdoctoral Fellow. This research was supported by USPHS Grant GM 1597 (to S. J. Singer).

‡ It is also possible that a residue on the protein is the photoactive species, and that a "reverse" photoaffinity labeling of the bound reagent occurs.

§ It is well known that the 3 OH of the cardiac glycoside aglycones allows the greatest variation in substitution without compromising binding to $(Na^+ + K^+)$ adenosinetriphosphatase. For this reason, the carbohydrate end of the molecule may be more exposed to the solvent.

¶ One unfortunate circumstance is that $(Na^+ + K^+)$ adenosine triphosphatase is itself inactivated by photolysis. This inactivation can be partially protected by performing the photolysis under nitrogen, suggesting that a photo-oxidation is occurring. For this reason, as well as the low level of labeling, correlation between labeling and irreversible loss of enzyme activity was not possible in these experiments.

1. Kyte, J. (1972) *J. Biol. Chem.* **247**, 7642–7649.
2. Singer, S. J. (1967) "Covalent labeling of active sites," in *Advan. in Protein Chem.* **22**, 1–54.
3. Hokin, L. E., Mokotoff, M. & Kupchan, S. M. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 797–804.
4. Ruoho, A. E., Hokin, L. E., Hemingway, R. J. & Kupchan, S. M. (1968) *Science* **159**, 1354–1355.
5. Ruoho, A. E., Blaiklock, R. & Hokin, L. E. (1973) submitted to *Mol. Pharmacol.*
6. Knowles, J. R. (1972) "Photogenated reagents for biological receptor-site labeling," in *Accounts Chem. Res.* **5**, 155–160.
7. Stefanovsky, Y. & Westheimer, F. H. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1132–1136.
8. Fleet, G. W. J., Knowles, J. R. & Porter, R. R. (1972) *Biochem. J.* **128**, 499–508.
9. Guthrow, C. E., Rasmussen, H., Brunswick, D. J. & Cooperman, B. S. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3344–3346.
10. Weygand, F., Bestmann, H. J. & Fritsche, H. (1960) *Chem. Ber.* **93**, 2340.
11. Jacobs, W. A. & Hoffmann, A. (1926) *J. Biol. Chem.* **67**, 609–619.
12. Vaughan, R. & Westheimer, F. H. (1969) *Anal. Biochem.* **29**, 305–310.
13. Kyte, J. (1971) *J. Biol. Chem.* **246**, 4157–4165.
14. Choules, G. L. & Zimm, B. H. (1965) *Anal. Biochem.* **13**, 336–344.
15. Matsui, H. & Schwartz, A. (1968) *Biochim. Biophys. Acta* **151**, 655–663.
16. Albers, R. W., Fahn, S. & Koval, G. J. (1963) *Proc. Nat. Acad. Sci. USA* **50**, 474–481.
17. Siegel, G. J., Koval, G. J. & Albers, R. W. (1969) *J. Biol. Chem.* **244**, 3264–3269.
18. Kyte, J. (1972) *J. Biol. Chem.* **247**, 7634–7641.
19. Shapiro, A. L., Vinuela, E. & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815–820.
20. Dunham, E. T. & Glynn, I. M. (1961) *J. Physiol.* **156**, 274–293.
21. Caldwell, P. C. & Keynes, R. D. (1959) *J. Physiol.* **148**, 8P–9P.
22. Kyte, J. (1971) *Biochem. Biophys. Res. Commun.* **43**, 1259–1265.
23. Whittam, R. (1962) *Biochem. J.* **84**, 110–118.
24. Kyte, J. (1974) *J. Biol. Chem.*, in the press.
25. Singer, S. J. & Nicholson, G. L. (1972) *Science* **175**, 720–731.
26. Martin, N., Warner, N. L., Roeder, P. E. & Singer, S. J. (1972) *Biochemistry* **11**, 4999–5005.
27. Singer, S. J., Martin, N. & Thorpe, N. (1971) *Ann. N.Y. Acad. Sci.* **190**, 342–351.
28. Ruoho, A. E., Kiefer, H., Roeder, P. E. & Singer, S. J. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2567–2571.
29. Hexter, C. S. & Westheimer, F. H. (1971) *J. Biol. Chem.* **246**, 3928–3933.