Light-Dependent Labeling of the Active Site of Sodium and Potassium Ion Activated Adenosinetriphosphatase with the Chromium Complex of 3′-*O*-[3-[(4-Azido-2-nitrophenyl)amino]-3-tritiopropionyl]adenosine 5′-Triphosphate†

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ABSTRACT: The synthesis of the chromium(III) coordination complex of 3′-*O*-[3-[(4-azido-2-nitrophenyl)amino]-3-tritiopropionyl]adenosine 5′-triphosphate ([^{3}H]-CrATPa) is described. When [^{3}H]-CrATPa was photolyzed in the presence of sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase], the reagent became attached irreversibly to the α-polypeptide of the enzyme. The amount of the photoaffinity reagent that was incorporated was decreased approximately 2-fold when excess ATP was added to the sample prior to photolysis, but an equivalent excess of AMP caused only a small decrease. The light-dependent reaction between the undirected parent compound, 3-[(4-azido-2-nitrophenyl)amino]-3-tritiopropionate, and the α-polypeptide was unaffected by the presence of ATP. It can be concluded that the incorporation of [^{3}H]-CrATPa that is sensitive to ATP is the product of a reaction occurring within the active site of (Na^+ + K^+)-ATPase. Native (Na^+ + K^+)-ATPase irradiated in the presence of either [^{3}H]-CrATPa alone or [^{3}H]-CrATPa and ATP was submitted to several different proteolytic digestions. The distribution of radioactivity among the fragments of the α-polypeptide produced in each digest was determined. It was found that the fragments [Castro, J., & Farley, R. A. (1979) *J. Biol. Chem. 254*, 2221] of apparent molecular weights 77 000, 58 000, and 40 000 displayed ATP-sensitive labeling but those of 41 000 and 35 000 did not. The specific labeling of the fragment of apparent molecular weight 40 000 places amino acids surrounding the active site at least 200 residues beyond the site of phosphorylation and suggests that the active site is formed from portions of the protein distant from each other in the primary structure.

Sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase][1] is the membrane-bound enzyme responsible for maintaining the gradients of sodium and potassium across the plasma membrane of the animal cell (Kyte, 1981a). Purified (Na^+ + K^+)-ATPase consists of two polypeptides, the α-polypeptide, which has an apparent molecular weight of 121 000 ± 6000, and a smaller glycoprotein, the β-polypeptide, which has an apparent molecular weight of 56 000 ± 7000 (Craig & Kyte, 1980). Several properties of the α-polypeptide have been reported. In native (Na^+ + K^+)-ATPase, the α-polypeptide is phosphorylated by ATP (Uesugi et al., 1971) during each turnover, binds cardiac glycosides (Ruoho & Kyte, 1974), contains sites to which antibodies can bind (Kyte, 1974), and has exposed sulfhydryl groups whose alkylation results in loss of enzymatic activity (Winslow, 1981). The function of the β-polypeptide is unknown.

One of the few methods for studying the molecular structure of (Na^+ + K^+)-ATPase relies on the covalent attachment of reagents to various distinct positions on the α-polypeptide and the subsequent identification of their location within the amino acid sequence. The research presented in this report represents one of the steps in the application of this approach to the region surrounding the portion of the active site to which ATP binds. The strategy outlined by Jeng & Guillory (1975) has been adopted. These authors described the synthesis of a set of photoaffinity analogues of ATP, each of which contains a nitrophenyl azide attached by an ester linkage to the ribose of ATP. Upon absorption of light, nitrophenyl azides are known to decompose to nitrenes, which are highly reactive and can form covalent bonds to several chemical species even though they display definite preferences (Ruoho et al., 1973; Reiser et al., 1968; Reiser & Leyshon, 1971; Smolinsky & Feuer, 1964). Therefore, it was proposed that, under the appropriate conditions of photolysis, any one of these ATP analogues might form covalent bonds to distinct positions in the vicinity of a site that, under normal circumstances, would bind ATP (Jeng & Guillory, 1975).

The first ATP analogue of the series described by Jeng & Guillory, 3′-*O*-[3-[(4-azido-2-nitrophenyl)amino]propionyl]-adenosine 5′-triphosphate (arylazido-β-alanyl-ATP),[1] has been modified and used on (Na^+ + K^+)-ATPase (Munson, 1981). Arylazido-β-alanyl-ATP was found to be a substrate of (Na^+ + K^+)-ATPase, a fact which would complicate the interpretation of the photolytic experiments. So that hydrolysis of the reagent by the enzyme could be prevented, the chromium(III) coordination complex of arylazido-β-alanyl-ATP (CrATPa)[1] was synthesized (Munson, 1981). In the dark, CrATPa is a reversible, competitive inhibitor of (Na^+ + K^+)-ATPase and is not hydrolyzed by the enzyme. When the enzyme was

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[1] Abbreviations: (Na^+ + K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); arylazido-β-alanyl-ATP, 3′-*O*-[3-[(4-azido-2-nitrophenyl)amino]propionyl]adenosine 5′-triphosphate; CrATPa, chromium(III) coordination complex of arylazido-β-alanyl-ATP; [^{3}H]-arylazido-β-alanyl-ATP, 3′-*O*-[3-[(4-azido-2-nitrophenyl)amino]-3-tritiopropionyl]adenosine 5′-triphosphate; [^{3}H]-CrATPa, the chromium(III) coordination complex of [^{3}H]-arylazido-β-alanyl-ATP; [^{3}H]-arylazido-β-alanine, 3-[(4-azido-2-nitrophenyl)-amino]-3-tritiopropionate; EDTA, ethylenediaminetetraacetic acid; Na-DodSO_4, sodium dodecyl sulfate; buffer A, 1 mM EDTA and 25 mM imidazolium chloride, pH 7.5.

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irradiated in the presence of the reagent, however, it was inactivated irreversibly. No inactivation was observed if excess ATP was added to the preparations before photolysis. Furthermore, the kinetics of the inactivation are inconsistent with a mechanism involving a simple biomolecular reaction between the enzyme and the reagent, but they do fit a function that can be derived from a mechanism in which CrATPa inactivates ($Na^+ + K^+$)-ATPase only while bound at the active site as a competitive inhibitor (Munson, 1981; Kyte, 1981b). The dissociation constant for the binding of CrATPa (8 μM) could be extracted from the dependence of fraction of inactivation (α_{E-A}^{∞}) on the initial concentration of CrATPa, and a similar value (9 μM) was observed for the dissociation constant of the reagent in the dark by direct titration. It was concluded from these observations that the light-dependent inactivation of ($Na^+ + K^+$)-ATPase by CrATPa is caused by the covalent attachment of the reagent at the active site of the enzyme (Munson, 1981).

In this report, the synthesis of a tritiated derivative of CrATPa, the chromium(III) complex of 3′-*O*-[3-[(4-azido-2-nitrophenyl)amino]-3-tritiopropionyl]adenosine 5′-triphosphate ([^{3}H]-CrATPa),[1] and experiments examining the light-dependent incorporation of this compound into ($Na^+ + K^+$)-ATPase are presented. The undenatured, modified enzyme has been submitted to several different tryptic and chymotryptic cleavages. On the basis of these experiments, conclusions regarding the location of the active site within the structure of the α-polypeptide of ($Na^+ + K^+$)-ATPase are discussed.

Experimental Procedures

Materials. Dimethylformamide was distilled over phthalic anhydride and stored over molecular sieves. Sodium dodecyl sulfate ($NaDodSO_4$)[1] from Sigma Chemical Co. was recrystallized (Burgess, 1969). Other chemicals obtained from this supplier were Na_2ATP (grade I, vanadate free) and trypsin inhibitor. 3-Aminopropionate was obtained from Eastman Organic Chemicals; carbonyldiimidazole was from Aldrich Chemical Co.; phenylmethanesulfonyl fluoride was from CalBiochem; α-chymotrypsin and trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone were from Worthington Biochemical Corp.; Protosol, Liquifluor, and 3-amino[3-^{3}H]propionate were from New England Nuclear; Dowex 50W-X2 (H^+) was from Bio-Rad Laboratories.

Synthesis of 3-[(4-Azido-2-nitrophenyl)amino]-3-tritiopropionate ([^{3}H]-Arylazido-β-alanine).[1] All of the synthetic procedures were carried out in dim light or darkness. To 5 mCi of 3-amino[3-^{3}H]propionate (40 mCi μmol^{-1}) dissolved in 5.0 mL of H_2O was added a 20 molar excess of 3-aminopropionate (0.25 mg, 2.8 μmol). The solution was lyophilized and the residue dissolved in 35 μL of 0.33 M Na_2CO_3 (11.6 μmol). A solution (100 μL) of 110 mM 4-fluoro-3-nitrophenyl azide (11.0 μmol) in ethanol was added and the mixture stirred magnetically overnight at 60 °C in a thermostated oil bath in a tube (8 mm × 70 mm) to which a water-cooled condensor (15 mm × 110 mm) was attached. The solvent was removed under a gentle stream of argon, and the residue was dissolved in 100 μL of H_2O and extracted with 300 μL of ether 9 times to remove unreacted, excess 4-fluoro-3-nitrophenyl azide. The material dissolved in the aqueous phase was acidified carefully with 1 M HCl until the orange-red [^{3}H]-arylazido-β-alanine precipitated. The product, in its neutral form, was removed from the aqueous phase by nine 300-μL extractions with ether. The ether was evaporated, and the [^{3}H]-arylazido-β-alanine was dried at room temperature in a desiccator over P_2O_5 (80% yield).

Synthesis of 3′-O-[3-[(4-Azido-2-nitrophenyl)amino]-3-tritiopropionyl]adenosine 5′-Triphosphate ([^{3}H]-Arylazido-β-alanyl-ATP).[1] [^{3}H]-Arylazido-β-alanine (2.2 μmol) was dissolved in 20 μL of dry *N,N*-dimethylformamide and added to 8.1 mg of carbonyldiimidazole (50 μmol) in a flame-dried test tube (6 mm × 50 mm) equipped with a small magnetic stirring bar. The mixture was stirred for 15 min, 100 μL of 130 mM Na_2ATP (13 μmol) was added, and the final solution was stirred for 2 h at room temperature. [^{3}H]-Arylazido-β-alanyl-ATP was isolated in a manner identical with that described earlier for nonradioactive arylazido-β-alanyl-ATP (Munson, 1981), using an analytical, thin-layer chromatogram (20 cm × 5 cm, 0.25 mm silica layer) (29% yield).

Synthesis of [^{3}H]-CrATPa. To 0.40 mL of a 0.75 mM solution of [^{3}H]-arylazido-β-alanyl-ATP that had been acidified to pH 3 with 0.3 M HCl and heated to 75 °C was added 0.4 mL of 0.85 mM $Cr(H_2O)_6Cl_3$ at the same temperature. The reaction was kept between 70 and 80 °C for 20 min, then cooled to room temperature, and immediately absorbed onto a small column containing 150 μL of settled Dowex 50W-X2 (H^+). The column was rinsed with 8 mL of H_2O, and [^{3}H]-CrATPa was eluted isocratically with 0.3 M pyridinium acetate, pH 3.8. The fractions containing the product were pooled, immediately extracted with 30 mL of ether 6 times to remove the pyridinium acetate, and concentrated to 125 μL by rotary evaporation. This concentrated solution was stored at −80 °C (17% yield on this step; 4% overall yield of [^{3}H]-CrATPa, based on 3-amino[3-^{3}H]propionate).

Purified [^{3}H]-CrATPa was submitted to chromatography on a thin-layer, silica plate with a solvent mixture of butanol, acetic acid, and H_2O (5:2:3). The product migrated on the chromatogram as a single, visible spot (R_f 0.60). Upon autoradiography of the plate, a heavily exposed area on the film was observed in the position of the product. No other regions on the film were exposed.

The following calculation was used to estimate the specific radioactivity of the product. Commercially obtained, nonradioactive 3-aminopropionate (zwitterionic solid) was carefully weighed and added to water to make a solution of a given molarity. A specific volume of this solution containing a 20 molar excess was mixed with the 3-amino[3-^{3}H]propionate from the supplier and the mixture brought to dryness. The residue was dissolved in a known volume and a small sample submitted to scintillation. From the total mass of 3-aminopropionate and the cpm, the specific radioactivity of the final 3-amino[3-^{3}H]propionate could be calculated in cpm per mole. This value then was used to calculate all molar concentrations of [^{3}H]-CrATPa mentioned subsequently in this report. When less valuable samples of [^{3}H]-CrATPa with lower specific radioactivities were used, it was found that this method of estimating concentrations yielded values within 15% of those calculated directly from the A_{460}, assuming an extinction coefficient of 5900 (Jeng & Guillory, 1975).

Preparation of ($Na^+ + K^+$)-ATPase for Irradiation with [^{3}H]-CrATPa. Purified ($Na^+ + K^+$)-ATPase was prepared from canine renal outer medulla as previously described (Munson, 1981) with the exception that 10 mM 2-mercaptoethanol was present throughout the purification. In the final step of the procedure, the enzyme, suspended in 1.0 mL, was separated from the 2-mercaptoethanol by centrifugation (SW 60 rotor, 250000*g*, 90 min) through 3.5 mL of 0.44 M sucrose dissolved in buffer A [1 mM ethylenediaminetetraacetic acid (EDTA),[1] 25 mM imidazolium chloride, pH 7.5].[1] This step was necessary since it was found that 10 mM 2-mercaptoethanol inhibited 90% of the specific incor-

poration of [^{3}H]-CrATPa into $(Na^+ + K^+)$-ATPase. The pellet was resuspended with 0.25 M sucrose in buffer A, using a glass tissue homogenizer. The final protein concentration of the enzyme preparaton was 10 mg mL^{-1}, based on the method of Lowry et al. (1951).

The same centrifugation procedure was utilized in several experiments to remove noncovalently bound [^{3}H]-CrATPa from irradiated membranes. After centrifugation, consecutive fractions (1 mL) were removed carefully from the top to the bottom of each centrifuge tube. When the radioactivity in these fractions and in the pellet was measured, it could be shown that in each case the unbound radioactive reagent had been separated quantitatively from the membranes by this method.

Solutions to be irradiated were prepared in the dark. An appropriate volume of 460 μM [^{3}H]-CrATPa (2.2×10^{12} cpm mmol^{-1}) was added directly to the concentrated $(Na^+ + K^+)$-ATPase and the solution mixed by vortexing. To half of the preparation was added enough Na_2ATP, dissolved in buffer A, to give the final concentrations of ATP, [^{3}H]-CrATPa, and enzyme indicated. An equal volume of buffer A was added to the other half of the sample.

Photolysis Procedure. Solutions to be irradiated were sealed into glass capillaries (20-μL Van-Lab micropipets) of uniform bore (0.700 mm i.d. and 1.2 mm o.d.) by plugging the tips with petroleum jelly. The loaded tubes were affixed to a glass plate (1 mm × 2.5 cm × 20 cm) and lowered into an iced water bath in a 600-mL beaker equipped with a stir bar. A tin-plated cylinder (21 cm × 13 cm), with a hole 7.5 cm in diameter cut into its side, was placed over the beaker to increase the light flux through the samples by reflection. The light source, a General Electric 150-V, 600-W, tungsten halogen lamp in a parabolic reflector 8 cm in diameter, was moved to a position 1 cm in front of the hole in the cylinder, directly in line with the samples, and 3.0 cm from them. While the lamp was on, the water bath was stirred continuously. Photolyses were for 8 min unless otherwise stated.

Specific Radioactivities. Measurements of tritium cpm values always were made in a scintillation solution containing a mixture of Protosol, Liquifluor, and toluene (5:5:100). For determination of the distribution of radioactivity in the polyacrylamide gels, each 2-mm slice was swelled by incubation in 8 mL of this scintillation solution for a period of 48 h at 50 °C prior to counting (Drickamer, 1976). It was determined, from the time course of cpm released, that greater than 90% of the radioactive reagent cast within a $NaDodSO_4$–polyacrylamide gel not submitted to electrophoresis could be detected by using this procedure. For the gels described in this report, a similar rate of release of cpm was observed for all slices regardless of the position in the gel from which they were derived, and it was concluded that all of the labeled peptides eluted from the slices at the same rate.

The specific radioactivities of the labeled polypeptides were calculated in the following manner. After the samples were submitted to electrophoresis, the gels were stained, clarified, and then scanned for absorbance at 525 nm. The absorbance peaks were integrated, and the radioactivity above background associated with the polypeptide was determined by measuring the tritium cpm in each 2-mm section of the gels. Specific radioactivity is presented as the total specific cpm within a given peak divided by the area, in centimeters squared, of the peak on the chart paper (cpm cm^{-2}). All gels in a given series were scanned at the same settings on the spectrophotometer and recorder.

Table I: Radioactivity Bound to Membranes and α-Polypeptide after Irradiation of $(Na^+ + K^+)$-ATPase in the Presence of either [^{3}H]-CrATPa or [^{3}H]-CrATPa and ATP[a]

	incorporation of ^{3}H (total cpm × 10^{-3})			
photolysis	washed membranes	supernatant from wash	α-polypeptide (unstained gel)	α-polypeptide (stained gel)
−ATP	83	126	14.0	10.0
+ATP	73	134	6.1	3.8
difference	10	−8	7.9	6.2

[a] A solution (200 μL) containing 30 μM [^{3}H]-CrATPa (2.2×10^{12} cpm mmol^{-1}), 30 μM $(Na^+ + K^+)$-ATPase (concentration of sites for the binding of ATP), and 250 mM sucrose in buffer A was split, and ATP was added to one of the halves to a final concentration of 220 μM. The two samples were irradiated simultaneously. Equivalent amounts (90 μg of protein) of each were diluted and then split into three equal parts (50 μl, 30 μg of protein). One part, washed membranes, was diluted to 0.5 mL with 150 μM ATP in buffer A, and the membranes were collected by centrifugation through 3.5 mL of 0.44 M sucrose. The other two were submitted directly to electrophoresis on $NaDodSO_4$-polyacrylamide gels. One pair of gels was sliced unstained and the other, after staining. The slices were submitted to scintillation counting. The position of the α-polypeptide was determined from mobility measured on the stained gel. Only the total cpm values above background associated with the α-polypeptides are reported.

Digestion of Native, Labeled $(Na^+ + K^+)$-ATPase. Samples of $(Na^+ + K^+)$-ATPase that had been modified by irradiation in the presence of [^{3}H]-CrATPa or, in the control, [^{3}H]-CrATPa and ATP were diluted separately with buffer A to a protein concentration of 1 mg mL^{-1}. All of the samples were brought to the same final Na_2ATP concentration of 22 μM. To 50 μL of these solutions was added either 10 μL of buffer A, 10 μL of 900 mM KCl in buffer A, or 10 μL of 60 mM $MgCl_2$ and 2.5 mM strophanthidin in buffer A. After a 5-min preincubation at 37 °C, 0.8 μg of trypsin or 5.6 or 20 μg of chymotrypsin was added to the appropriate samples to give the final concentrations listed. The digests with trypsin were quenched by adding 3 μg of trypsin inhibitor while chymotryptic digestions were halted by adding 60 μg of phenylmethanesulfonyl fluoride dissolved in 1 μL of dioxane. The samples were made 2% in $NaDodSO_4$ and submitted to electrophoresis on $NaDodSO_4$–polyacrylamide gels 8% in acrylamide [acrylamide–*N,N'*-methylenebis(acrylamide), 37:1] by the procedure of Swank & Munkres (1971).

Results

Light-Dependent, Covalent Incorporation of [^{3}H]-CrATPa into $(Na^+ + K^+)$-ATPase. A solution containing $(Na^+ + K^+)$-ATPase and [^{3}H]-CrATPa (2.2×10^{12} cpm mmol^{-1}) was prepared in which the final concentrations of active sites and radioactive reagent were both 30 μM, respectively. A control sample, to which ATP was added, was made from half of the mixture to distinguish incorporation of reagent at the active site from that occurring elsewhere on the protein. These two preparations were sealed in capillary tubes and irradiated in a simple apparatus for 8 min.

Because the identification of the labeled polypeptides in these samples would require submitting each of them to electrophoresis on $NaDodSO_4$–polyacrylamide gels, it had to be determined if the covalently incorporated, radioactive reagent would remain attached to the enzyme under the conditions of this procedure. After the photolysis equal amounts from each preparation were either homogenized in the presence of ATP and collected by centrifugation or submitted directly to electrophoresis (Table I). It was observed

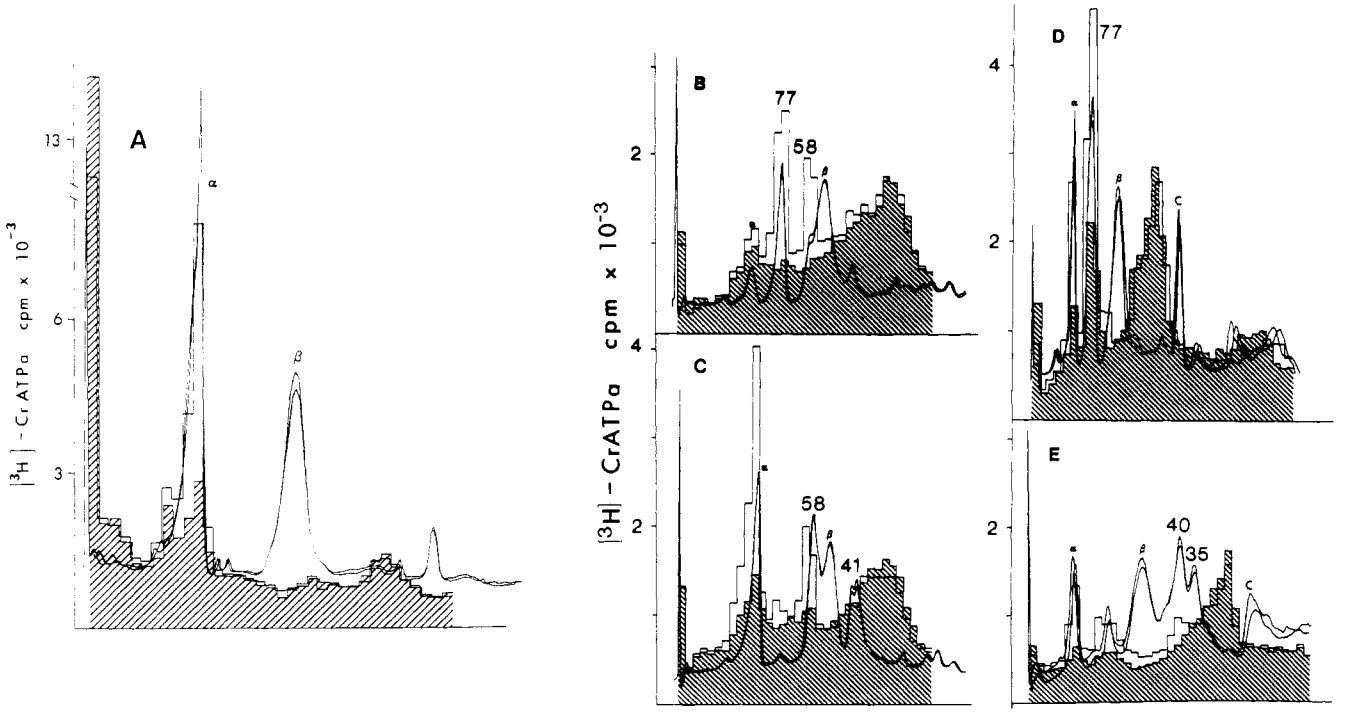

FIGURE 1: Labeling of the α-polypeptide and its fragments with [^{3}H]-CrATPa. Photolyzed preparations, described in Table I, were either submitted directly to electrophoresis on $NaDodSO_4$–polyacrylamide gels as a control (A) or diluted and digested with trypsin or chymotrypsin in the native state prior to preparation for electrophoresis (B–E). In each panel, scans of protein absorbance (A_{525}) from gels of unprotected samples and samples protected with ATP are superimposed. The radioactivity profiles from both gels are presented also, as bar graphs, and the areas corresponding to the cpm in samples irradiated in the presence of ATP are shaded. (A) No digestion; (B) digested with 13 μg mL^{-1} trypsin for 20 min; (C) same trypsin treatment but in the presence of 150 mM KCl; (D) digested with 92 μg mL^{-1} chymotrypsin for 20 min; (E) digested with 320 μg mL^{-1} chymotrpysin for 40 min in the presence of 10 mM $MgCl_2$ and 0.4 mM strophanthidin. The chymotrypsin peak C is indicated in panels D and E. All digestions were performed at 37 °C. All samples were in buffer A and contained 3 μM remaining, photolyzed CrATPa, 22 μM Na_2ATP, and 1 mg mL^{-1} enzyme. Undigested enzyme also was incubated at 37 °C for 20 min prior to electrophoresis.

that a significant fraction (40%) of the total radioactivity could not be removed by the simple washing procedure from the membranes irradiated with the reagent alone. In the sample containing ATP, however, slightly less [^{3}H]-CrATPa (35%) had become bound. In both cases, all of the radioactivity initially present (2.08×10^5 cpm) could be accounted for in the supernatant and the pellet. If the enzyme was mixed with [^{3}H]-CrATPa that had been previously photolyzed alone or if the enzyme and reagent were mixed but not irradiated, only minor amounts of radioactivity, 3.2% and 0.6% of the total, respectively, remained attached to the membranes after homogenization and centrifugation of the samples.

When equivalent samples were submitted to electrophoresis, the only component of molecular weight greater than 10000 that showed significant incorporation of radioactivity was the α-polypeptide of ($Na^+ + K^+$)-ATPase (Figure 1A). The total radioactivity above background in the region of the α-polypeptide, however, was a minor fraction of that which had remained bound to the membranes during washing (Table I). Nevertheless, most (80%) of the difference in incorporation caused by the addition of ATP prior to photolysis, the quantity that defines specific modification, was still associated with the α-polypeptide in the unstained polyacrylamide gels. Another set of identical polyacrylamide gels was stained for protein and clarified in a solution of 7.5% acetic acid and 5.0% methanol (pH 2.3). These latter gels contained a much lower level of radioactivity in the background (600 cpm in each gel slice) compared with that of the unstained gels (1600 cpm in each gel slice), but most of the specific incorporation associated with the α-polypeptide (80% of that in the unstained gel) was retained. On the basis of this experiment, a decision was made to follow the labeling of ($Na^+ + K^+$)-ATPase by submitting samples routinely to electrophoresis followed by staining. The ability to determine the relative protein concentration of the α-polypeptide from the scan of the stained gel more than compensates for the decrease in levels of modification (<40%). If it is assumed that ATP only prevents the incorporation of the reagent that occurs at the active site, it can be estimated, from the ATP-dependent difference in radioactivity associated with the membranes, that a minimum of 5% of the α-polypeptides in this experiment were specifically labeled.

To demonstrate that incorporation of the reagent was complete at the end of 8 min of irradiation, an experiment was performed in which samples of ($Na^+ + K^+$)-ATPase (10 μM) and [^{3}H]-CrATPa (19 μM) were irradiated for 8, 16, or 24 min. It was found that photolysis longer than 8 min did not increase the incorporation of the reagent into the α-polypeptide, either in the presence or in the absence of ATP (350 μM), and a photolysis of 8 min was used in all subsequent experiments. On the other hand, when samples were not irradiated prior to electrophoresis, no peaks of radioactivity above background (<5% of the total cpm observed on the α-polypeptide from irradiated samples) could be detected in the gels.

The specificity of the incorporation of [^{3}H]-CrATPa is defined by the effect of the competing ligand, ATP. In the stained gels described above (Table I), it was found that the specific radioactivity above background of the α-polypeptide from enzyme irradiated with [^{3}H]-CrATPa alone was 980 cpm cm^{-2}, but addition of ATP to the solution prior to photolysis reduced this value to 420 cpm cm^{-2}, a decrease of 57%. In other experiments, in which the concentrations of enzyme and [^{3}H]-CrATPa ranged from 10 to 16 μM and from 16 to 18 μM, respectively, the incorporation of the reagent into the enzyme was measured and compared with that which was

observed in the presence of either a 10-fold molar excess of AMP or a 10-fold excess of ATP. The decrease in the specific radioactivity of the α-polypeptide produced by the addition of AMP to the irradiated mixture (15 ± 3%) was 3–4-fold lower than the decrease (48 ± 5%) produced by ATP. No further decrease in incorporation was observed with as much as a 30-fold molar excess of ATP over [^{3}H]-CrATPa, whereas an equimolar concentration of ATP was sufficient to produce a 40% decrease. Finally, a sample was irradiated in which [^{3}H]-arylazido-β-alanine was substituted for [^{3}H]-CrATPa. A solution of $(Na^+ + K^+)$-ATPase (34 μM) and [^{3}H]-arylazido-β-alanine (32 μM, 2.2×10^{12} cpm mmol^{-1}) was irradiated by using the standard procedure. Half of the sample also contained ATP (230 μM). The specific radioactivity of the α-polypeptide (480 cpm cm^{-2}) was decreased only 7% by the presence of ATP and was similar in magnitude to that of samples irradiated under similar conditions with [^{3}H]-CrATPa (2.2×10^{12} cpm mmol^{-1}) but protected by ATP.

It can be concluded from all of these experiments that about 50–60% of the [^{3}H]-CrATPa covalently bound to the α-polypeptide in the absence of ATP is inserted into the protein while the reagent is occupying the active site of the enzyme (Munson, 1981). The remaining 40–50% presumably is inserted elsewhere on the accessible surface of the native enzyme in a nonspecific, random fashion.

Specific Digestion of $(Na^+ + K^+)$-ATPase Labeled with [^{3}H]-CrATPa. Following irradiation in the presence of [^{3}H]-CrATPa, modified $(Na^+ + K^+)$-ATPase was submitted to digestion with trypsin or chymotrypsin while the enzyme was in its native state (Jørgensen, 1975; Giotta, 1975). After the appropriate proteolytic inhibitor was added, the digested enzyme was denatured with $NaDodSO_4$ and submitted to electrophoresis on $NaDodSO_4$–polyacrylamide gels. The gels were stained to locate the fragments and sliced for scintillation counting. In preliminary experiments with unmodified enzyme, conditions had been adjusted so that sets of fragments identical with those defined previously (Castro & Farley, 1979) were produced. In all of these experiments, the distributions of radioactivity among the fragments generated from $(Na^+ + K^+)$-ATPase irradiated in the presence of [^{3}H]-CrATPa and ATP, as a control for nonspecific labeling, were compared with the patterns given by cleavage of $(Na^+ + K^+)$-ATPase irradiated with the reagent alone. Representative results from three separate experiments of this type are shown in Figure 1. These are from two tryptic (Figure 1B,C) and two chymotryptic (Figure 1D,E) digests of the same labeled $(Na^+ + K^+)$-ATPase preparation described in Table I.

Cleavage of the native enzyme by either protease in the presence of a low concentration of ATP (22 μM) produced a comparatively high yield of large fragment with an apparent molecular weight of 77 000[2] (Castro & Farley, 1979; Figure 1B,D). In the presence of ATP (22 μM) and a high concentration of KCl (150 mM), the α-polypeptide was split by trypsin into two fragments with apparent molecular weights of 58 000 and 41 000 (Castro & Farley, 1979; Figure 1C). A smaller yield of these two fragments also was observed in samples cleaved by trypsin in the presence of ATP (Figure 1B). Finally, in the presence of $MgCl_2$ (10 mM), ATP (22 μM), and strophanthidin (0.4 mM), the α-polypeptide was cleaved by chymotrypsin to the fragment of M_r 77 000 which then was split rapidly into fragments with apparent molecular weights of 40 000 and 35 000 (Castro & Farley, 1979; Figure 1E). The locations of each of these fragments in the overall sequence of the α-polypeptide has been defined (Castro & Farley, 1979).

[2] All references to specific masses refer to the designations of apparent mass used by Castro & Farley (1979).

Table II: Specific Radioactivities of Polypeptides Labeled with [^{3}H]-CrATPa[a]

polypeptide[b]	specific radioactivity[c] −ATP	+ATP	difference
α-polypeptide (uncleaved control)	940 ± 80[d] (3)[e]	410 ± 80 (3)	530 ± 30 (3)
77-kilodalton fragment	850 ± 130 (3)	290 ± 50 (3)	560 ± 150 (3)

[a] Samples were submitted to electrophoresis as described in Table I and Figure 1. [b] As displayed in Figure 1. [c] ^{3}H values in cpm cm^{-2}. [d] Standard deviation. [e] Number of determinations.

It can be seen that the fragments of M_r 77 000 and 58 000 were labeled by [^{3}H]-CrATPa. Furthermore, the radioactivity associated with them was greatly diminished when ATP was present during the photolysis (Figure 1B–D). In contrast, the fragment of M_r 41 000 was not labeled significantly by the reagent (Figure 1C). Finally, although the fragment of M_r 40 000 produced by chymotryptic cleavage in the presence of strophanthidin displayed a minor amount of labeling (Figure 1E), the radioactivity in the region of the fragment of M_r 35 000 on the same gel showed no ATP-dependent decrease. Treatment of the labeled enzyme preparation with chymotrypsin in the presence of strophanthidin also produced, albeit in very low yield, two previously unreported fragments that migrated in the gels with apparent molecular weights of 68 000 and 79 000, respectively. The significant peaks of radioactivity associated with these fragments were decreased markedly by the presence of ATP in the irradiated sample (Figure 1E).

A large, broad peak of radioactivity was found in gels of both cleaved and uncleaved, labeled enzyme (Figure 1). The source of the peak, which migrated in a region below the β-polypeptide, was not identified. It was present in gels of samples that had been precipitated with trichloroacetic acid or passed over Sephadex G-25 prior to electrophoresis (data not shown). The amount of this material was highly variable but appeared to increase following digestion of the samples. Nevertheless, incorporation of radioactivity into this component never responded significantly to the addition of ATP to the samples.

The specific radioactivities (cpm cm^{-2}) of some of the polypeptides shown in Figure 1 were calculated from this experiment and several others. Means and standard deviations of these values are presented in Table II.

Finally, an experiment was performed in which the preparations of $(Na^+ + K^+)$-ATPase, described earlier, that had been labeled with [^{3}H]-arylazido-β-alanine, were submitted to digestion by trypsin followed by electrophoresis. The cleavages were performed under the same conditions as those described in Figure 1. As before, a large, broad peak of radioactivity was observed in the region below the β-polypeptide. This peak was similar in magnitude and location to that seen in the gels of enzyme labeled with [^{3}H]-CrATPa. None of the fragments of the α-polypeptide formed in the cleavages of these preparations displayed decreased labeling in the presence of ATP.

Discussion

A procedure has been described for the synthesis of [^{3}H]-CrATPa, a radioactive photoaffinity analogue of ATP. On a thin-layer chromatogram, the purified, radioactive reagent migrated as a single, red spot with an R_f value identical with that of nonradioactive CrATPa (Munson, 1981). Upon

autoradiography of the plate the only radioactivity detected was in the position of the spot. These observations and the protocol for the synthesis are considered sufficient to identify the new compound.

A photolysis procedure was developed for the purpose of incorporating this radioactive reagent into $(Na^+ + K^+)$-ATPase. A significant fraction of the [^{3}H]-CrATPa photolyzed in the presence of the enzyme could no longer be washed from the particulate fraction of the preparation (Table I). As a control, it was shown that no reagent was bound to unirradiated membranes. Presumably, attachment of [^{3}H]-CrATPa to the enzyme requires light-dependent conversion of the aryl azide to the aryl nitrene (Munson, 1981). Nitrenes are known to be extremely reactive, and it seems reasonable to assume that the activated reagent became bound covalently to the membranes.

A decrease in the radioactivity attached to the membranes was observed upon addition of ATP to the mixture that was irradiated. The majority of this ATP-dependent decrease was shown to be accounted for by a decrease in the level of radioactivity covalently bound to the α-polypeptide of $(Na^+ + K^+)$-ATPase (Table I and Figure 1A). It has been demonstrated previously that CrATPa, when photolyzed in the presence of $(Na^+ + K^+)$-ATPase, irreversibly inactivates the enzyme by a covalent reaction that occurs while it is noncovalently bound to the active site. This inactivation is prevented by the addition of ATP (Munson, 1981). Although the inactivation of $(Na^+ + K^+)$-ATPase by [^{3}H]-CrATPa in the present experiments was too low to be measured accurately,[3] it seems reasonable to assume that the incorporation of radioactivity which was sensitive to ATP was the product of this specific modification reaction. This conclusion is reinforced by two other observations. First, addition of a 10-fold excess of AMP to the photolysis led to a much smaller decrease in the incorporation of the reagent into the α-polypeptide than addition of ATP. This is consistent with the observation that the dissociation constant for binding of AMP to the enzyme is at least 100-fold larger than that observed for ATP (Moczydlowski & Fortes, 1981) or CrATPa (Munson, 1981). Therefore, AMP would not be expected to compete effectively with the reagent for the active site under the experimental conditions that were used. Second, the nucleotide portion of [^{3}H]-CrATPa was required for the ATP-dependent decrease in incorporation. The parent compound that lacks this functionality, [^{3}H]-arylazido-β-alanine, was incorporated into the α-polypeptide with an efficiency that was similar to that given by [^{3}H]-CrATPa (480 cpm cm^{-2} against 410 $\pm$ 80 cpm cm^{-2}, respectively) in the presence of ATP, and the incorporation of [^{3}H]-arylazido-β-alanine was not significantly decreased by addition of ATP.

All of these results, considered together, demonstrate that the α-polypeptide of $(Na^+ + K^+)$-ATPase contains the active site for ATP hydrolysis. This conclusion has been drawn previously from the observations that the α-polypeptide is phosphorylated during turnover of the enzyme (Uesugi et al., 1971) and that a general thiocarbamoylating agent, fluorescein 5′-isothiocyanate, inactivates the enzyme by modifying the α-polypeptide in a reaction whose rate is decreased by addition of ATP (Karlish, 1980; Carilli et al., 1982).

The incorporation of [^{3}H]-CrATPa, which was detected in these experiments by isolation of the product of its reaction, amounted to about 5% of the available active sites. This is significantly less than the maximum modification ($\alpha_{E\text{-}A}^{\infty}$ = 0.4) achieved in the examination of the inactivation of $(Na^+ + K^+)$-ATPase by this reagent (Munson, 1981). These earlier experiments were performed with a 30–50-fold excess of reagent over active sites. In the case of the labeling experiments described here, it had been assumed that such a large excess of reagent would result in unmanageable incorporation elsewhere on the enzyme at positions which are unrelated to the active site. This expectation was substantiated by the observation that significant nonspecific incorporation into the α-polypeptide occurred even under stringent, equimolar circumstances (Figure 1A). The concentration of the enzyme was therefore held approximately equal to that of the radioactive reagent and 3-fold higher than its dissociation constant (Munson, 1981). The ability to concentrate the protein defined this final level.

It can be calculated, using the results and equations of the earlier titration measurements (Munson, 1981; Kyte, 1981b), that, at these concentrations of enzyme and [^{3}H]-CrATPa, about 60% of the reagent present at any instant during the photolysis is noncovalently bound at the active site of $(Na^+ + K^+)$-ATPase. It was observed that 35% of the reagent present became covalently attached to the membranes at locations other than the active site (Table I). Because most of the free reagent became attached to them, it appears that the membranes are much more efficient nitrene scavengers (Ruoho et al., 1973) than the solvent. The majority of the reagent reacted with components of the membranes other than protein (Table I).

The results obtained here indicate that at least 5% of the total amount of reagent added became incorporated into the portion of the protein forming the active site. Since 60% of the reagent is noncovalently bound to the active site at any instant, the partition coefficient (k_i/k') between insertion of the reagent into amino acid residues surrounding that site and other nonproductive reactions is about 0.1.

Although this is significantly smaller than the values for this parameter that were required to explain the kinetics of inactivation observed in earlier experiments (Munson, 1981; Kyte, 1981b), the concentrations of protein and lipid in the present reactions were very different form those employed earlier. Since these two components consumed a large fraction of the nitrene (Table I), direct comparisons between these two investigations would be of little consequence. It is possible that hydrolysis of the ester linkage in the reagent prior to photolysis may have contributed to the lower than expected level of specific incorporation.

In the native enzyme the region that surrounds the active site and into which [^{3}H]-CrATPa is inserted in 9 $\pm$ 2 Å from the ribose ring and 14 $\pm$ 3 Å from the γ-phosphate of the reagent. These distances were derived from measurements made on a Corey–Pauling–Kendrew model of CrATPa. The assumption was made that, while [^{3}H]-CrATPa is bound to the active site, the CrATP portion of the reagent is held fixed with respect to the mass of the protein by noncovalent interactions, whereas the aryl azide is allowed free movement.

The model also can be used to estimate the maximum number of amino acid residues that the bound reagent can reach. The distance from the ribose ring to the azide was measured for all possible orientations of the arylammonium propionate, and these vectors defined the bounded surface that is accessible to the azide. This surface was adjusted to the proper scale and moved within a three-dimensional model of a typical soluble protein, lactate dehydrogenase from the dogfish. It was found that, at a maximum, portions of six amino acid residues could lie within this surface. Therefore,

[3] K. Munson, unpublished observation.

it seems likely that all specific incorporation of [^{3}H]-CrATPa occurs at fewer than seven positions within the sequence of the α-polypeptide.

Under carefully controlled conditions, involving the manipulation of the concentrations of substrates and inhibitors, the α-polypeptide of native $(Na^+ + K^+)$-ATPase is cleaved by trypsin or chymotrypsin at well-defined locations to yield certain fragments derived form specific regions of the overall amino acid sequence (Jørgensen, 1975; Giotta, 1975; Castro & Farley, 1979). It is believed that the apparent changes in the sensitivity to cleavage at each of these locations occur because the enzyme assumes distinct conformational states whose relative stabilities are dependent upon which of the ligands are present.

The location of a certain modification can be identified by an examination of the distribution of radioactivity among the fragments of the α-polypeptide obtained under these various cleavage conditions (Castro & Farley, 1979; Farley et al., 1980; Carilli et al., 1982; Jørgensen et al., 1982; Karlish et al., 1977). The labeling of the α-polypeptide that was sensitive to ATP and that occurred from within the active site resulted in the covalent incorporation of [^{3}H]-CrATPa into an amino acid residue or residues contained within the 77-kilodalton,[2] the 58-kilodalton, and the 40-kilodalton fragments but missing from the 41-kilodalton fragment (Figure 1). While no labeling of the 35-kilodalton fragment was detected, it cannot be stated unequivocally that it does not contain a site of modification.

The 77-kilodalton fragment appears to contain all of the specific labeling that was associated with the α-polypeptide. The specific radioactivity of the 77-kilodalton fragment that is sensitive to the addition of ATP was greater than that of the α-polypeptide by an amount consistent with the loss of about 15–20% of unlabeled mass (Table II). Parenthetically, it was observed that the specific radioactivity of the 77-kilodalton fragment derived from the sample protected by ATP was significantly less than that of the α-polypeptide in the corresponding control (Figure 1 and Table II). One explanation for this unexpected result would be that the NH_2-terminal region of the α-polypeptide contains an amino acid side chain that is particularly reactive toward nitrenes. This reminds one of results obtained earlier with 5-[^{125}I]iodonaphthyl 1-azide, a reagent that also is converted into a nitrene upon absorption of light (Bercovici & Gitler, 1978). It has been suggested (Karlish et al., 1977) that nearly all of this reagent is incorporated into a region within approximately 100 amino acid residues of the NH_2-terminal end of the α-polypeptide when it is photolyzed in the presence of $(Na^+ + K^+)$-ATPase. This observation is consistent with the existence of a particularly reactive scavenger in this region of the sequence.

Although it was not possible to determine accurately the specific radioactivity of the 58-kilodalton fragment because it overlapped the β-polypeptide of the enzyme during electrophoresis, this polypeptide also was labeled heavily and specifically by [^{3}H]-CrATPa (Figure 1C). On the other hand, the 41-kilodalton fragment, derived during the production of the 58-kilodalton fragment, was not labeled.

A small but significant amount of specific incorporation was found associated with the 40-kilodalton fragment (Figure 1E). Although the results are difficult to interpret quantitatively, it is apparent from an examination of Figure 1 that only a minority of the ATP-sensitive labeling associated with the 58-kilodalton fragment (Figure 1C) was retained by the 40-kilodalton fragment (Figure 1E). Since the 40-kilodalton fragment is derived from the COOH-terminal end of the 58-kilodalton fragment and yet displayed so little specific labeling, one might suggest that the majority of the specific incorporation of [^{3}H]-CrATPa occurred on the section of the α-polypeptide between the NH_2-terminal end of the 40-kilodalton fragment and the NH_2-terminal end of the 58-kilodalton fragment. But no radioactivity ever was detected on the 35-kilodalton fragment (Figure 1E) despite the fact that, taken together, the 35-kilodalton fragment and the 40-kilodalton fragment are believed to comprise a section of the α-polypeptide that includes all of the 58-kilodalton fragment (Castro & Farley, 1979). There are several ways to resolve this paradox.

From the stioichiometry of the incorporation (<5%), it is clear that each labeled α-polypeptide was modified at only one position and that the majority (>90%) of the α-polypeptides were not labeled at all. Therefore, it is possible that labeled α-polypeptides behave differently from the majority of the protein present in the digestion. During the photolysis, the reagent may have partitioned among several amino acids surrounding the active site owing to the fact that the nitrene is located at the end of the arylammonium propionate arm. During the steps between the photolysis and the digestion, the ester linkage within the bound reagent may have hydrolyzed in a significant fraction of the modified enzyme molecules, and this could have released the CrATP portion of [^{3}H]-CrATPa. From these consideratons it follows that several chemically distinct, singly labeled α-polypeptides, mixed with a much larger amount of unmodified enzyme entered the digestion.

If incorporation into the 40-kilodalton region of the α-polypeptide was an infrequent event, this would explain the low specific radioactivity of this fragment (Figure 1E). Alternatively, all of the modification could have occurred in this region, but some of the labeled α-polypeptides might have become more resistant to cleavage by chymotrypsin than the unlabeled α-polypeptides. In fact, there were two unusual, large fragments present on this gel, a 68-kilodalton fragment and a 79-kilodalton fragment. These fragments were present in the cleavages of both the sample irradiated in the presence of ATP and the sample irradiated in its absence. Therefore, they are not peculiar to α-polypeptide that has been modified at the active site. Nevertheless, since they contained 50% of the total specific labeling other than that of the α-polypeptide but by mass were produced in very low yield, they appear to be preferred products of the cleavage of specifically modified α-polypeptide. They could have contained the missing, modified 40-kilodalton fragments. It also is possible, however, that some or all of these resistant fragments bore radioactivity incorporated into the region of the sequence common to the 58-kilodalton fragment and the 35-kilodalton fragment.

That these large fragments were observed at all appears to be due to the presence of low levels of ATP in the cleavage mixtures.[3] It is well-known that the binding of cardiac glycosides and ATP to $(Na^+ + K^+)$-ATPase is an antagonistic process; one would expect that enzyme with [^{3}H]-CrATPa covalently bound within the region of the active site would bind strophanthidin poorly. This may be one of the reasons that the labeled 40-kilodalton fragment was produced in such a low yield.

Unfortunately, this consideration raises the possibility that the modified enzyme, bearing a covalently attached nucleotide, might assume a conformation whose pattern of cleavage is completely unique. This would put into question the conclusion that the radioactivity which comigrated with the 40-kilodalton fragment was attached to a fragment derived from that region of the sequence. The fact, however, that radioactivity was

found comigrating with the 79-kilodalton fragment, the 68-kilodalton fragment, and the 40-kilodalton fragment, all products of the normal cleavages, argues that the specifically modified enzyme molecules were being cleaved at the same locations as the unmodified molecules, albeit at different rates. Furthermore, by including the addition of exogenous ATP to all of the samples, the protocol for the digestion had been designed purposely to minimize the peculiar affects of the covalently bound nucleotide.

Finally, when a summation is made of all of the radioactivity arising from specific incorporation across a gel such as that shown in Figure 1E, only about half of the specific cpm found in the control (Figure 1A) can be accounted for. It is possible that very small peptides, which arose from the region in the α-polypeptide between the 35-kilodalton fragment and the 40-kilodalton fragment and which contained significant amounts of incorporated [^{3}H]-CrATPa, were lost. In fact, the occurrence of incorporation into this particular location between the two fragments would explain both the loss of radioactivity, owing to production of small peptides from this hinge region, and the appearance of the partially cleaved fragments of 68-kilodaltons and 79-kilodaltons, owing to direct steric interference with the digestion that occurs at this hinge region.

When all of these possiblities are considered, it appears to be the case that a significant portion, if not the majority, of the specific incorporation of [^{3}H]-CrATPa into the α-polypeptide of ($Na^+ + K^+$)-ATPase occurred in regions of the sequence beyond the COOH-terminal end of the 35-kilodalton fragment. This would place amino acid residues surrounding the active site at least 200 residues away from the aspartyl phosphate, which is known to be contained within the 41-kilodalton fragment (Castro & Farley, 1979; Allen et al., 1980).

The distribution of incorporation of [^{3}H]-CrATPa among the various fragments of the α-polypeptide is very similar to that seen with fluorescein 5′-isothiocyanate (Carilli et al., 1982). This thiocarbamoylating agent, which is supposed to incorporate at only one position within the sequence of the α-polypeptide (Karlish, 1980), also is attached to the 58-kilodalton fragment. A substantial yield of a novel fragment, resulting from interrupted cleavage, bearing fluorescence, and having an apparent molecular weight similar to the 68-kilodalton and the 79-kilodalton fragments observed in this study, also was seen[cf. Figure 2D (Carilli et al., 1982)]. Finally, significant amounts of fluorescence were cleaved from the protein during digestion with chymotrypsin in the presence of ouabain. The similarities between the results obtained with fluorescein 5′-isothiocyanate and [^{3}H]-CrATPa strongly suggest that these two disparate reagents react with the same region of the protein. This provides support for the proposal (Karlish, 1980) that incorporation of fluorescein 5′-isothiocyanate occurs at the active site.

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Registry No. ATPase, 9000-83-3; [^{3}H]-arylazido-β-alanyl-ATP, 84863-72-9; [^{3}H]-arylazido-β-alanine, 84863-73-0; 3-amino[3-^{3}H]-propionic acid, 66185-53-3; 4-fluoro-3-nitrophenyl azide, 28166-06-5; ATP, 56-65-5.

References

Allen, G., Trinnaman, B. J., & Green, N. M. (1980) *Biochem. J. 187*, 591.
Bercovici, T., & Gitler, C. (1978) *Biochemistry 17*, 1484.
Burgess, R. R. (1969) *J. Biol. Chem. 244*, 6168.
Carilli, C. T., Farley, R. A., Perlman, D. M., & Cantley, L. C. (1982) *J. Biol. Chem 257*, 5601.
Castro, J., & Farley, R. A. (1979) *J. Biol. Chem. 254*, 2221.
Craig, W. S., & Kyte, J. (1980) *J. Biol. Chem. 255*, 6262.
Drickamer, L. K. (1976) *J. Biol. Chem. 251*, 5115.
Farley, R. A., Goldman, D. W., & Bayley, H. (1980) *J. Biol. Chem. 255*, 860.
Giotta, G. J. (1975) *J. Biol. Chem. 250*, 5159.
Jeng, S. J., & Guillory, R. J. (1975) *J. Supramol. Struct. 3*, 448.
Jørgensen, P. L. (1975) *Biochim. Biophys. Acta 401*, 399.
Jørgensen, P. L., Karlish, S. J. D., & Gitler, C. (1982) *J. Biol. Chem. 257*, 7435.
Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111.
Karlish, S. J. D., Jørgensen, P. L., & Gitler, C. (1977) *Nature (London) 269*, 715.
Kyte, J. (1974) *J. Biol. Chem. 249*, 3652.
Kyte, J. (1981a) *Nature (London) 292*, 201.
Kyte, J. (1981b) *J. Biol. Chem. 256*, 3231.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Moczydlowski, E. G., & Fortes, P. A. G. (1981) *J. Biol. Chem. 256*, 2346.
Munson, K. B. (1981) *J. Biol. Chem. 256*, 3223.
Reiser, A., & Leyshon, L. J. (1971) *J. Am. Chem. Soc. 93*, 4051.
Reiser, A., Willets, F. W., Terry, G. C., Williams, V., & Marley, R. (1968) *Faraday Soc. Trans. 64*, 3265.
Ruoho, A. E., & Kyte, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2352.
Ruoho, A. E., Kiefer, H., Roeder, P. E., & Singer, S. J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2567.
Smolinsky, G., & Feuer, B. I. (1964) *J. Am. Chem. Soc. 86*, 3085.
Swank, R. T., & Munkres, K. (1971) *Anal. Biochem. 39*, 462.
Uesugi, S., Dulak, N. C., Dixon, J. F., Hexum, T. D., Dahl, J. L., Perdue, J. F., & Hokin, L. E. (1971) *J. Biol. Chem. 246*, 531.
Winslow, J.W. (1981) *J. Biol. Chem. 256*, 9522.