Reaction of (Na–K)ATPase with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole: Evidence for an Essential Tyrosine at the Active Site†

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ABSTRACT: The reaction of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole [NBD-Cl] with purified eel electroplax Na^+ and K^+ stimulated adenosine triphosphatase [(Na–K)ATPase] has been monitored by changes in the (Na–K)ATPase activity, the K^+ stimulated *p*-nitrophenyl phosphatase [PNPase] activity, and the protein ultraviolet absorption spectrum. The NBD-Cl reacts with two tyrosine residues per mol of enzyme (~6–7 nmol/mg of protein), as judged by changes in protein absorption spectra and incorporation of [^{14}C]NBD-Cl. The modified tyrosine groups are located on the M_r = 95 000 polypeptide chain and react at different rates. Only one tyrosine modification is necessary for complete inhibition of (Na–K)ATPase activity, although both must be modified for complete inhibition of PNPase activity. Reversal of these modifications by 2-mercaptoethanol restores 65% of both activities. Na^+ increases the rate of tyrosine modification, K^+ decreases the rate, and ATP affords the more reactive tyrosine group complete protection. NBD-Cl modification of ~6–7 nmol of tyrosine groups/mg of protein results in a large decrease in ATP affinity as judged by equilibrium binding. These results are compared with similar results obtained from NBD-Cl modification of the coupling factors of oxidative phosphorylation and photophosphorylation. A model is presented suggesting an asymmetric arrangement of two 95 000 polypeptide chains with a single tyrosine residue at the ATP site.

The (Na–K)ATPase[1] is the enzyme responsible for maintaining cation gradients in most eucaryotic cells. It has been purified from a number of sources including mammalian kidney (Kyte, 1971; Jørgensen, 1974a), mammalian brain (Nakao et al., 1974), eel electric organ (Hokin, 1974), the dogfish rectal gland (Hokin et al., 1973), and the duck salt gland (Hopkins et al., 1976). The most highly purified preparations contain two polypeptide chains. The largest chain (M_r = 95 000) forms an aspartate–phosphate anhydride bond as an intermediate in ATP hydrolysis. The smaller polypeptide chain is a glycoprotein with a molecular weight of approximately 50 000. Enzyme purified from dog kidney, containing only these two polypeptide chains and some native lipid, has been reconstituted into synthetic lipid vesicles and is capable of active sodium and potassium transport with an efficiency of approximately 3 Na^+ transported in and 2 K^+ transported out per ATP hydrolyzed on the outside of the vesicle (Goldin, 1977).

A number of inestigators have examined the molecular weight of the active enzyme using radiation inactivation, ouabain binding stoichiometry, ATP binding stoichiometry, and phosphorylation stoichiometry (for review, see Jørgensen, 1975b). Recent experiments with highly purified enzyme suggest minimal molecular weights of 250 000 to 330 000 based on ATP and ouabain binding capacities (i.e., 3–4 nmol of ouabain or ATP sites/mg of protein; Lane et al., 1973; Jørgensen, 1974b). Cross-linking experiments demonstrate that the enzyme is an oligomer containing two 95 000 polypeptide chains and two glycoprotein molecules (Giotta, 1976; Kyte, 1975).

Covalent modifications designed to investigate the active site of the (Na–K)ATPase have indicated the presence of a cysteine group (Patzelt-Wenczler et al., 1975; Hart and Titus, 1973; Shoot et al., 1977), an arginine group (de Pont et al., 1977), and a tyrosine group (Masiak and D'Angelo, 1975). However, these investigators have not been able to demonstrate a preparation of enzyme in which the modifications are specific to and stoichiometric with the active site.

The reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) has been used to modify sulfhydryl and tyrosine groups on a number of enzymes. The identity of the group modified may be determined by the absorption spectrum of the reacted protein. This reagent has been used extensively to investigate a highly reactive tyrosine group found in a number of coupling factors of oxidative phosphorylation and photophosphorylation (Ferguson et al., 1974a,b, 1975a,b; Deters et al., 1975; Nelson et al., 1974). The reactive tyrosine group has been located with respect to other ligand binding sites on the chloroplast coupling factor CF_1 (Cantley and Hammes, 1975a,b, 1976a,b). Kuriki et al. (1976) found that small amounts of this reagent were capable of inactivating the (Na–K)ATPase. In this paper we report a detailed investigation of the modification of purified eel electroplax (Na–K)-ATPase with NBD-Cl. The identification, stoichiometry, and subunit location of reacted residues are determined by protein

† From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138. *Received September 7, 1977.* This work was supported by Grant HL 08893 from the National Institutes of Health and Grant BMS 73-06752 from the National Science Foundation.

‡ National Institutes of Health Postdoctoral Fellow (GM 05508-01).

§ Supported by Harvard University Faculty Aide Program.

¶ National Institutes of Health Postdoctoral Fellow (AM 00899-02).

[1] Abbreviations used: (Na–K)ATPase, Na^+ and K^+ stimulated adenosine triphosphatase; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PNPase, K^+ stimulated *p*-nitrophenylphosphatase; EDTA, ethylenediaminetetraacetic acid; F_1, mitochondrial coupling factor 1; CF_1, chloroplast coupling factor 1; BF_1, the bacterial inner membrane ATPase; NBD-reacted-enzyme, (Na–K)ATPase that has been reacted with NBD-Cl as described in the Experimental Section; NBD-Tyr-enzyme, (Na–K)-ATPase containing 6–7 nmol of NBD-Cl modified tyrosine residues per mg of protein; reactivated enzyme, (Na–K)ATPase that has been reacted with NBD-Cl and reactivated by incubation in 2-mercaptoethanol; NBD-cysteine, NBD-Cl reacted with the sulfhydryl moiety of a cysteine residue; NBD-tyrosine, NBD-Cl reacted with the phenolic oxygen of a tyrosine residue.

0006-2960/78/0417-0418$01.00/0

absorbance changes and radioactive labeling. The effects of NBD-Cl modification on ATPase and PNPase activities give additional information about the structure and function of the native enzyme.

Experimental Section

Materials. L-Norepinephrine and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. NBD-Cl was from Eastman. The [^{3}H]ATP was from New England Nuclear and was purified by paper chromatography using isobutyric acid/ammonia/water (100/8/52; v/v/v). The [^{14}C]NBD-Cl was from Research Products International. ATP was purchased as the Sigma Grade from Sigma Chemical Co. and purified by passage through a Chelex-100 column (Josephson and Cantley, 1977). This procedure removes an inhibitor of the (Na-K)ATPase which produces a time-dependent decay of enzyme activity under most assay conditions (Cantley and Josephson, 1976; Fagan and Racker, 1977).

(Na-K)ATPase was prepared from the electric organ of the electric eel. The first stage of the purification involved the preparation of microsomes by a modification of the method of Albers et al. (1963). Sixty grams of electric organ and 300 mL of a buffer consisting of 20 mM Tris-Cl, 2 mM EDTA, 1 mM dithiothreitol (pH 7.4) were mixed in a Waring blender for 2 min. All steps were performed at 0–4 °C in this buffer. The mixture was centrifuged for 20 min at 9000*g*. The pellet was mixed with 200 mL of buffer and homogenized by hand in a Potter-Elvejhem device until a uniform suspension was obtained. Forty-milliliter portions of the suspension were then sonicated with a Branson 360 sonicator, medium tip, level 4 intensity with 20 sonications of 0.5 s each. The suspension was continuously stirred by a magnetic stir bar during sonication. The mixture was centrifuged as above and the supernatants from both centrifugations were pooled and centrifuged for 60 min at 50 000*g*. The pellet was mixed with 120 mL of buffer, homogenized, and sonicated as above. The resulting mixture was applied to discontinuous sucrose gradients and spun in an IEC A-192 rotor for 4 h at 50 000*g*. The lower sucrose solution contained 10 mL/tube of 37% w/v sucrose (1.14 g/mL) and the upper solution contained an equal volume of 29% w/v sucrose (1.10 g/mL). Fifteen milliliters of protein suspension was applied to the top of each tube. Separation occurs on the basis of density; electric organ (Na-K)ATPase has a density similar to the kidney preparation described by Jørgensen (1974a). (Na-K)ATPase activity was highest at the interface of the two gradients. Gradients were fractionated from the top by means of peristaltic pump and fraction collector. Enzyme was concentrated by dilution in buffer followed by centrifugation for 1.5 h at 50 000*g*. The pellet was then collected, homogenized, sonicated, and applied to the sucrose gradients as above. Following the second sucrose gradient, enzyme was concentrated and stored at −70 °C after quick freezing in a dry ice–acetone mixture. This procedure yields about 15 mg of enzyme with a specific activity of 14–16 μmol/(mg min) at 37 °C. A gel of the purified material is shown in Figure 4A. Although no detergents are employed in the purification procedure, the membrane fragments obtained are sufficiently fine to pass through Sephadex G-50 coarse and permit spectral measurements at 1 mg/mL concentration.

Kinetic Measurements. All optical density changes and absorption spectra were measured in a Cary 14 recording spectrophotometer with a thermostated cell holder. (Na-K)ATPase activities were measured using a coupled assay (Cantley and Josephson, 1976). All assays were done at 37 °C in the presence of 100 mM NaCl, 25 mM KCl, 3 mM ATP, 5 mM $MgCl_2$, 20 mM Hepes–triethylamine (pH 7.4), 1.4 mM phosphoenolpyruvate, 0.26 mM NADH, 10 μg/mL pyruvate kinase, 10 μg/mL lactic dehydrogenase, and approximately 1 μg/mL (Na-K)ATPase.

The *p*-nitrophenyl phosphatase (PNPase) activities were measured by the change in optical density at 410 nm. All assays were done at 37 °C in the presence of 20 mM Hepes–triethylamine (pH 7.4), 25 mM KCl, 2.5 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$, 2.5 mM L-norepinephrine, 50 μM dithiothreitol, and approximately 1–5 μg/mL (Na-K)ATPase. The L-norepinephrine was added to maintain the enzyme in an active state since the eel electroplax enzyme undergoes a norepinephrine-reversible inhibition in the presence of Mg^{2+} and K^+ and absence of Na^+ much like the dog brain (Na-K)ATPase (Josephson and Cantley, 1977).

Reactions with NBD-Cl. NBD-Cl reactions with the (Na-K)ATPase were carried out at 25 °C in the presence of 100 μM NBD-Cl, 20 mM Tris-Cl (pH 7.4), 2 mM EDTA, 0.1–0.5 mg/mL enzyme, and other ligands specified. The NBD-Cl was added from a 10 mM solution in dimethyl sulfoxide freshly prepared. The concentration of NBD-Cl was reduced by less than 10% during the reaction time. ATPase and PNPase activities were followed by periodic extraction of 10- or 20-μL aliquots from the reaction mixture, which were then added to 1 mL of the assay mixtures described above. Initial velocities were measured within 20 s of enzyme addition. In experiments where the NBD-Cl modifications were followed by changes in the optical density of the reaction mixture, the reference solution was identical with the reaction mixture but lacked enzyme.

The NBD-reacted-enzyme was prepared by incubating 100 μM NBD-Cl with 1–2 mg/mL (Na-K)ATPase in 20 mM Tris-Cl (pH 7.4), 2 mM EDTA for 20 min. The reaction was stopped by cooling to 4 °C, centrifuging at 50 000*g* for 20 min, and resuspending the pellet in 20 mM Tris-Cl (pH 7.4), 2 mM EDTA.

The NBD-Tyr-enzyme or [^{14}C]NBD-Tyr-enzyme was prepared by reacting NBD-Cl or [^{14}C]NBD-Cl with 1–2 mg/mL (Na-K)ATPase under the above conditions and then stopping the reaction by the addition of 2-mercaptoethanol to a final concentration of 2%, incubating for 5 min at 25 °C, and passing the mixture through a (1 cm × 23 cm) Sephadex G-50 coarse column equilibrated with 100 mM NaCl, 5 mM Na_2HPO_4 (pH 7.4). The effluent was monitored at 280 nm by passing it through a microflow cell in a Cary 15 spectrophotometer. An excellent separation of protein from small molecules was achieved as judged by 280-nm absorbance and radioactivity of fractions eluted. In some experiments, the protein solution from this column was solubilized in 1% sodium dodecyl sulfate and passed through a Sephadex G-50 fine column (1 cm × 25 cm) equilibrated with 25 mM Tris-Cl (pH 7.2), 0.1% sodium dodecyl sulfate to separate any noncovalently bound radioactive material from the protein. However, this procedure gave no additional separation of radioactivity from the protein and caused additional protein aggregation in electrophoresis.

The reactivated-enzyme was prepared by the same procedure as the NBD-Tyr-enzyme except that the 2-mercaptoethanol incubation was extended to 90 min.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the general procedure of Weber and Osborn (1969). 6% gels were prepared by adding 1 mL of 0.15% ammonium persulfate; 30 μL of redistilled tetramethylenediamine; 9 mL of 0.2 M sodium phosphate (pH 7.0), 0.2% sodium dodecyl sulfate; and 4 mL of H_2O to 6 mL of acrylamide solution (22.2 g acrylamide and 0.6 g of *N,N'*-methylenebisacrylamide per 100 mL of H_2O).

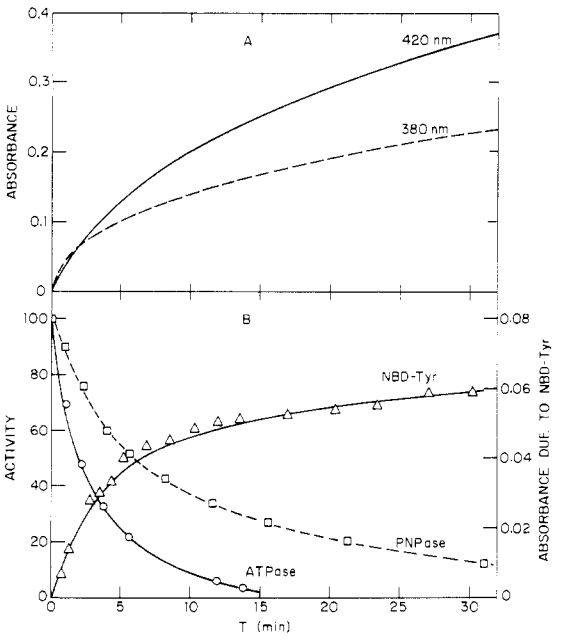

FIGURE 1: (A) The time-dependent increase in protein absorbance at 380 nm and 420 nm during reaction with 100 μM NBD-Cl in the presence of 20 mM Tris-Cl (pH 7.4), 2 mM EDTA, 25 °C as described in the Experimental Section. The (Na-K)ATPase concentration was 1 mg/mL. (B) The decrease in (Na-K)ATPase (○) and PNPase (□) activities during reaction with NBD-Cl under the same conditions described in A. The activities are relative to values measured prior to reaction as described in the Experimental Section. The absorbance due to NBD-tyrosine (△) was determined by subtracting 0.46 times the absorbance at 420 nm from the absorbance at 380 nm in A (see text). The solid line through the data was drawn using eq 4 and the parameters $a_{380} = 0.036/(\text{mg/mL})$, $k_1 = 0.26$ min^{-1} and $k_2 = 0.04$ min^{-1}.

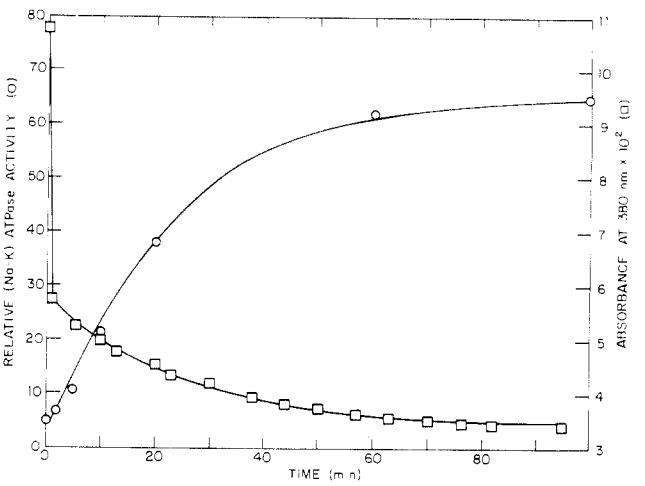

FIGURE 2: The reactivation of NBD-Cl inhibited enzyme by 2-mercaptoethanol as a function of time. At 0 time, the NBD-reacted-enzyme (see Experimental Section) was made 2% in 2-mercaptoethanol and the protein absorbance at 380 nm (□) and the (Na-K)ATPase activity (○) were monitored as described in the Experimental Section. The (Na-K)ATPase activity prior to NBD-Cl reaction was assigned a relative value of 100. The solution contained 20 mM Tris-Cl (pH 7.4), 2 mM EDTA, 0.32 mg/mL NBD-reacted-enzyme, 25 °C. For the absorbance measurement, the reference compartment of the Cary spectrophotometer contained 0.32 mg/mL of native (Na-K)ATPase in the same buffer.

Electrophoresis was performed in 0.1 M sodium phosphate (pH 7.0), 0.1% sodium dodecyl sulfate at 8 mA per gel.

Unstained gels were sliced into 2-mm sections, each of which was added to 4 mL of tissue solubilizer (40 mL Eastman Concentrate I and 50 mL Eastman tissue solubilizer diluted to 1 L with toluene), incubated at 50 °C for 24 h, and counted in a Beckman liquid scintillation counter.

Binding Experiments. Equilibrium [^{3}H]ATP binding measurements were made by the forced dialysis procedure of Cantley and Hammes (1973). Approximately 1-3 mg/mL of native enzyme or NBD-reacted-enzyme was incubated with varying concentrations of [^{3}H]ATP for 20 min at 25 °C in the presence of 20 mM Tris-Cl (pH 7.4), 2 mM EDTA (100 μL total volume). A small fraction of the solution (approximately 20 μL) was forced through a Amicon XM-50 membrane in a Metalloglass ultrafiltration cell by the application of approximately 30 psi of nitrogen. The radioactivities in 10 μL of filtrate and in 10 μL of solution before filtration were determined by liquid scintillation counting in 10 mL of Aquasol. The concentration of ATP bound was determined by subtracting the concentration of free ATP (in filtrate) from the total ATP concentration. A correction for ATP trapped in the membrane was made by measuring [^{3}H]ATP bound in the presence of 1 mM unlabeled ATP.

All protein concentrations were determined by the procedure of Lowry et al. (1951) in the presence of 1% sodium dodecyl sulfate.

Results

Evidence for an Essential Tyrosine Group. The reaction of the purified (Na-K)ATPase with NBD-Cl can be followed by the increase in absorbance at 420 nm or 380 nm, and the decrease in (Na-K)ATPase or *p*-nitrophenyl phosphate (PNPase) activities (Figure 1A,B). The PNPase activity is inhibited more slowly than the ATPase activity; however, the inhibition of either of these activities saturates more rapidly than the 420-nm absorbance increase. A sample of enzyme was reacted with NBD-Cl for 30 min, solubilized with sodium dodecyl sulfate, and passed through a Sephadex G-50 fine column, and its ultraviolet absorption spectrum was taken (see Experimental Section). This spectrum revealed an absorption peak at 420 nm, which is indicative of the NBD-cysteine adduct (see Figure 3A for the spectrum of NBD-*N*-acetylcysteine), and a stoichiometry of approximately 30 nmol of cysteine groups modified per mg of protein was established from the extinction coefficient of the NBD-cysteine moiety.

The absorbance at 380 nm is due to the products of NBD-Cl reaction with both cysteine and tyrosine groups. The 380-nm absorbance due to the tyrosine adduct alone may be estimated by subtracting 46% of the 420-nm absorbance from the 380-nm absorbance for each time point (the 380-nm absorbance/420-nm absorbance ratio of NBD-*N*-acetylcysteine is 0.46; Figure 3A). The NBD-Cl modification of tyrosine groups saturates at the same rate as the loss of PNPase activity (Figure 1B) suggesting that inactivation is due to this reaction. The stoichiometry of tyrosine groups modified after reaction for 30 min with NBD-Cl is approximately 6 nmol per mg of protein.

Further evidence for the involvement of tyrosine groups in inactivation is presented in Figures 2 and 3. Figure 2 shows the effect of 2-mercaptoethanol on NBD-reacted-enzyme. The 380 nm absorbance (and the 420-nm absorbance; data not presented) decreased rapidly during mixing time with 2-mercaptoethanol and then continued to decrease more slowly for the next 90 min. The slow decrease in absorption at 380 nm parallels an increase in (Na-K)ATPase activity (and PNPase activity; data not shown) suggesting that the slowly reversing class of sites modified by NBD-Cl is responsible for inactivation. Neither the ATPase nor the PNPase activity could be reversed more than 65% by this treatment, suggesting the presence of some form of irreversible modification.

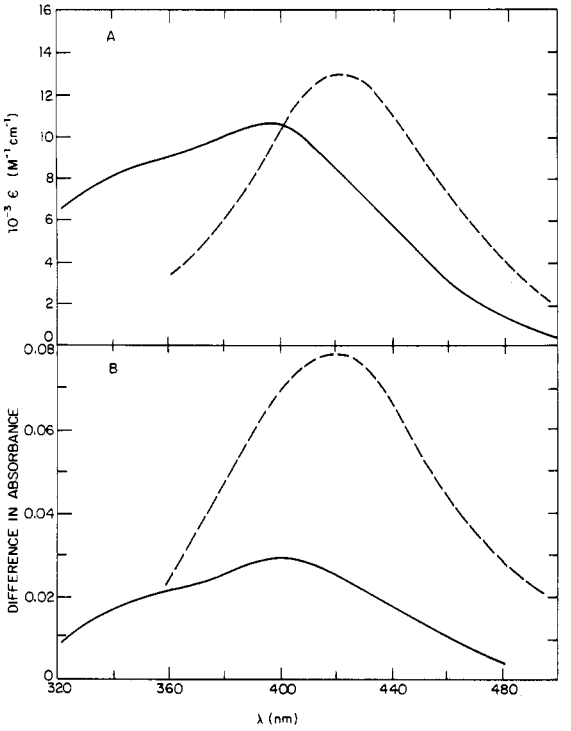

FIGURE 3: (A) The absorption spectra of NBD-tyrosine (solid line) and NBD-N-acetylcysteine (dashed line). The NBD-tyrosine spectrum was taken from Cantley and Hammes (1975b) and the NBD-*N*-acetylcysteine spectrum was from Birkett et al. (1970). (B) The difference in absorption spectra between NBD-reacted-enzyme before addition of 2% 2-mercaptoethanol and 2 min after addition of 2-mercaptoethanol (dashed line), and the difference between 2 min in 2-mercaptoethanol and 90 min in 2-mercaptoethanol (solid line). The spectra were taken from the experiment described in Figure 2.

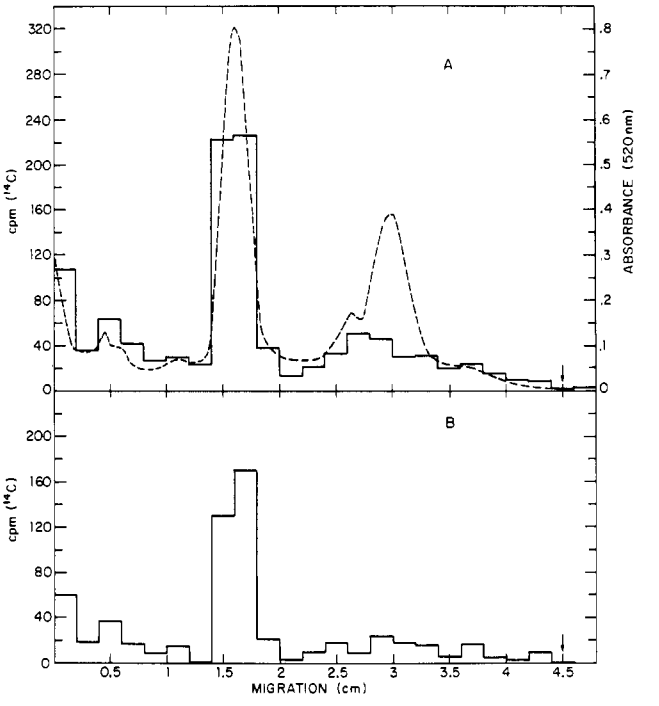

FIGURE 4: (A) A radiation profile of [^{14}C]NBD-Tyr-enzyme after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (solid line). The enzyme contained 10.8 nmol of NBD/mg of protein. The Coomassie Blue stain intensity (dashed line) is also presented. (B) The difference in radiation profiles between [^{14}C]NBD-Tyr-enzyme and ^{14}C-labeled reactivated-enzyme. The [^{14}C]NBD-Tyr-enzyme contained 10.8 nmol of [^{14}C]NBD/mg of protein, the ^{14}C-labeled reactivated-enzyme contained 4.8 nmol of [^{14}C]NBD/mg of protein and the two gels contained equal amounts of protein. The arrows represent the position of the tracking dye, bromophenol blue. See the Experimental Section for labeled enzyme preparations and electrophoresis procedures.

The identity of the modified groups was determined by measuring the absorption spectrum of the NBD-reacted-enzyme 2 min after 2-mercaptoethanol addition (i.e., after the rapid change in 380 nm absorbance is complete but before significant ATPase reactivation) and again 90 min after addition. The difference between the spectra taken at these two time points indicates that the groups reversed during reactivation are NBD-tyrosine residues (Figure 3B). The stoichiometry of NBD-tyrosine is approximately 7 nmol per mg of protein. This value is in good agreement with the number of tyrosine groups reacted in Figure 1B.

The difference between the spectrum of NBD-reacted-enzyme before and 2 min after 2-mercaptoethanol addition identifies the groups reversed during mixing time as NBD-cysteine residues (Figure 3B). The number of NBD-cysteine residues reduced during the mixing time was approximately 24 nmol per mg of protein demonstrating rapid and nearly complete reversal of the NBD-cysteine adducts.

Subunit Location of Essential Tyrosine Group. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of [^{14}C]NBD-Tyr-enzyme is presented in Figure 4A. The major Coomassie Blue stain band at 1.6 cm has a molecular weight of approximately 95 000; the band at 3.0 cm has a molecular weight of approximately 50 000 and contains carbohydrate as judged by Schiff–periodic acid staining. The peaks at high molecular weight are primarily due to 95 000 chain aggregation (2-mercaptoethanol was omitted from the electrophoresis system to avoid reversal of the NBD-Cl modification). Approximately 60% of the total stain and 75% of the total radioactivity is found in the 95 000 chain and higher aggregates. The total amount of NBD-Cl reacted is 10.8 nmol/mg of protein as judged by the specific activity of [^{14}C]NBD-Tyr-enzyme.

Further support for the location of the modified tyrosine is obtained by subtracting the radioactive profile of ^{14}C-labeled reactivated enzyme from that of [^{14}C]NBD-Tyr-enzyme (Figure 4B). This Figure reveals that >80% of the radioactivity removed during reactivation is on the 95 000 chain or higher aggregates. The stoichiometry of radioactive label removed during reactivation is 6.0 nmol/mg of protein, in good agreement with the results in Figures 2 and 3 based on the extinction coefficient of NBD-tyrosine (7 nmol/mg of protein). The reactivated enzyme contains approximately 4.8 nmol/mg of protein of radioactive label that is not reversed by 90-min treatment with 2-mercaptoethanol.

The stoichiometry of NBD-tyrosine in the 95 000 chain may also be estimated from the fraction of radioactive label (Figure 4B) and fraction of Coomassie Blue stained protein migrating at 1.6 cm. This number is 7.3 nmol/mg or approximately 0.7 mol of tyrosine residues per mol of 95 000 chain. Assuming the functional (Na-K)ATPase contains a dimer of 95 000 polypeptide chains then approximately 1.4 tyrosine residues per enzyme molecule are labeled by NBD-Cl in 20 min.

The Effect of Ligands on NBD-Cl Modification. Semilog plots of the (Na-K)ATPase and PNPase activities as a function of time during NBD-Cl reaction are presented in Figures 5A and B. The ATPase activity exhibits a single exponential decay under all conditions studied. Na^+ increases the rate of decay and K^+ slows down the decay. Increasing the ionic strength by adding 100 mM choline chloride to the Tris, EDTA buffer had no effect on the decay rate (data not presented).

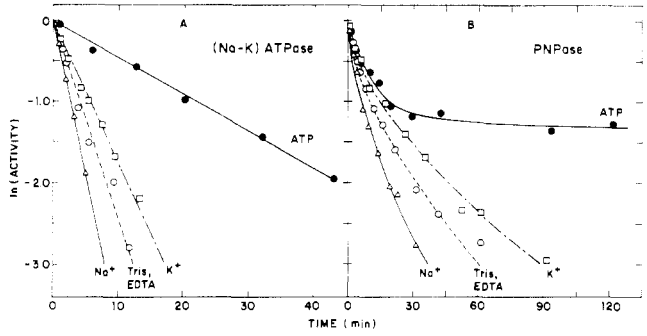

FIGURE 5: (A) The natural log of (Na-K)ATPase activity as a function of time during reaction with 100 μM NBD-Cl at 25 °C. All reaction mixtures contained 20 mM Tris-Cl (pH 7.4) and 2 mM EDTA. Where indicated, 100 mM NaCl (△), 20 mM KCl (□), or 100 μM ATP (●) were also present. (B) The natural log of PNPase activity as a function of time during reaction with 100 μM NBD-Cl. The conditions were the same as in A. The lines in A and B were drawn using eq 1 through 3 and the parameters in Table I.

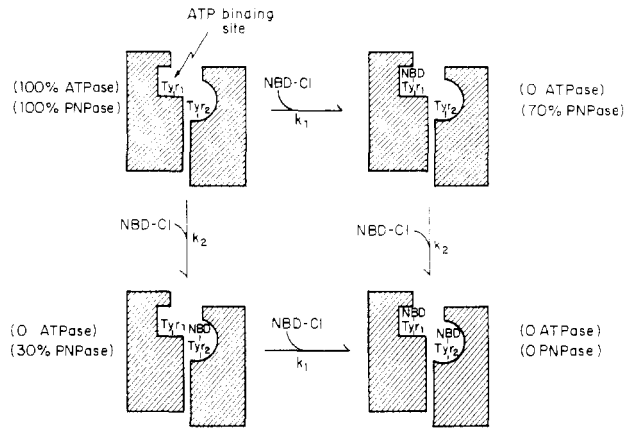

FIGURE 6: A model describing NBD-Cl modification of (Na-K)ATPase. Two tyrosine groups are located on asymmetrically associated 95 000 polypeptide chains. The more reactive tyrosine residue (Tyr_1) is indicated to be near the high affinity ATP binding site. k_1 and k_2 are the pseudo-first-order rate constants for NBD-Cl modification of Tyr_1 and Tyr_2, respectively. Modification of either site prevents (Na-K)ATPase activity; however, some PNPase activity still occurs if one site is unmodified.

Although Na^+ and ATP have been reported to stabilize the same enzyme conformational state as judged by tryptic digests and *N*-ethylmaleimide incorporation (Jørgensen, 1975a,b; Skou, 1974), they have opposing effects on the rate of NBD-Cl inactivation suggesting ATP protects by steric hindrance rather than by conformational change. The rate of ATPase inactivation in the presence of Mg^{2+}, Mg^{2+} + ATP, and Mg^{2+} + Na^+ + ATP was similar to that observed in the presence of ATP alone (data not presented).

The effect of NBD-Cl on PNPase activity cannot be described by a single exponential decay (Figure 5B). These results imply the requirement of more than one modification per enzyme molecule for complete inhibition of PNPase activity. As in the ATPase decay, Na^+ speeds up PNPase inactivation and K^+ slows it down. ATP offers protection of 30% of the PNPase activity from NBD-Cl inactivation for more than 2 h (notice the difference in abcissa scales for Figures 5A and B).

A Model Explaining NBD-Cl Reaction with Tyrosine Residues. The [^{14}C]NBD-Cl labeling experiments show that modification of approximately one tyrosine residue per 95 000 chain or two residues per enzyme molecule is sufficient for inactivation of ATPase and PNPase activities. The double exponential decay in PNPase activity (Figure 5B) suggests that these two tyrosine groups are reacting at different rates and the relatively rapid single exponential decay in ATPase activity (Figure 5A) suggests that only one of these tyrosine residues need be modified to fully inhibit this activity. These results may be explained by negative cooperativity in reaction of two identical tyrosine residues or by a preexisting asymmetry in subunit arrangement resulting in different reactivities for identical residues. (This latter assumption is mathematically identical to assuming two different tyrosine residues are reacting on the same subunit.) A model consistent with all the above data is presented in Figure 6 using the assumption of two asymmetric tyrosine residues independently reacting with NBD-Cl with pseudo-first-order rate constants k_1 and k_2. This model will accurately explain the data in Figure 5 if it is assumed that modification of either tyrosine group causes complete inhibition of (Na-K)ATPase activity but only partial inhibition of PNPase activity. The protection by ATP may be explained by assuming ATP binds near Tyr_1 blocking its reaction with NBD-Cl and thereby only allowing 70% inhibition of PNPase activity. Although this model is not unique, it is the simplest model consistent with the experimental data.

The model was tested by checking how well the rate constants k_1 and k_2 agree when determined independently from the decay in ATPase activity, the decay in PNPase activity, and the increase in protein absorbance at 380 nm. The value of $k_1 + k_2$ may be estimated from the decay in ATPase using eq 1 (derived from the model in Figure 6)

$$A_{(\text{ATPase})} = e^{-(k_1+k_2)t} \quad (1)$$

where $A_{(\text{ATPase})}$ is the fraction of ATPase activity remaining at time t. This equation was fit to the data in Figure 5A by a least-squares program and the best fit values of $k_1 + k_2$ are presented in Table I. In the presence of ATP, k_1 is assumed to be 0.

The individual values of k_1 and k_2 may be determined from the decay in PNPase activity using eq 2

$$A_{(\text{PNPase})} = e^{-(k_1+k_2)t} + (0.7k_1 + 0.3k_2) \times (e^{-k_1 t} - e^{-k_2 t})/(k_2 - k_1) \quad (2)$$

where $A_{(\text{PNPase})}$ is the fraction of PNPase activity remaining at time t. This equation was fit to the data in Figure 5B using a nonlinear least-squares computer program and the best fit values of k_1 and k_2 are presented in Table I. The PNPase decay in the presence of ATP was fit by eq 3 which assumes k_1 = 0.

$$A_{(\text{PNPase})} = 0.7e^{-k_2 t} + 0.3 \quad (3)$$

If the coefficients 0.7 and 0.3 in eq 2 and 3 were allowed to vary, the best fit values were within 10% of 0.7 and 0.3, respectively, for all the data in Figure 5B. The lines in Figures 5A and 5B were drawn using eq 1-3 and the parameters in Table I.

The sums of the rate constants ($k_1 + k_2$) determined by the two techniques are in good agreement (Table I) under all conditions studied offering excellent support for the model. The model is further supported by the increase in absorbance at 380 nm due to NBD-tyrosine formation in Figure 1B.

$$OD_{380} = a_{380}[2 - 2e^{-(k_1+k_2)t} - (k_1 + k_2)(e^{-k_1 t} - e^{-k_2 t})/(k_2 - k_1)] \quad (4)$$

These data were fit using eq 4 where a_{380} is the absorbance at 380 nm for a single NBD-tyrosine modification per enzyme. The value of k_2 = 0.04 min^{-1} was assumed from the PNPase

TABLE I: Pseudo-First-Order Rate Constants for NBD-Cl Reaction with Tyrosine Groups.[a]

Ligands[b]	k_1 (min^{-1})	k_2 (min^{-1})	$k_1 + k_2$ (min^{-1})	Assay
Tris, EDTA	0.18	0.04	0.22	PNPase, Figure 5B
Tris, EDTA	-	-	0.225	ATPase, Figure 5A
Tris, EDTA	0.26	0.04[c]	0.30	OD_{380}, Figure 1B
K^+	0.12	0.03	0.15	PNPase, Figure 5B
K^+	-	-	0.15	ATPase, Figure 5A
Na^+	0.25	0.07	0.32	PNPase, Figure 5B
Na^+	-	-	0.37	ATPase, Figure 5A
ATP	0	0.07	0.07	PNPase, Figure 5B
ATP	0	0.05	0.05	ATPase, Figure 5A

[a] The rate constants were calculated using eq 1-4 in the text derived from the model in Figure 6. [b] All reactions were done at 25 °C in the presence of 100 μM NBD-Cl, 20 mM Tris-Cl (pH 7.4), and 2 mM EDTA. Where present, the K^+, Na^+, and ATP concentrations were 20 mM, 100 mM, and 100 μM, respectively. [c] This value was assumed in the calculation of k_1 (see the text).

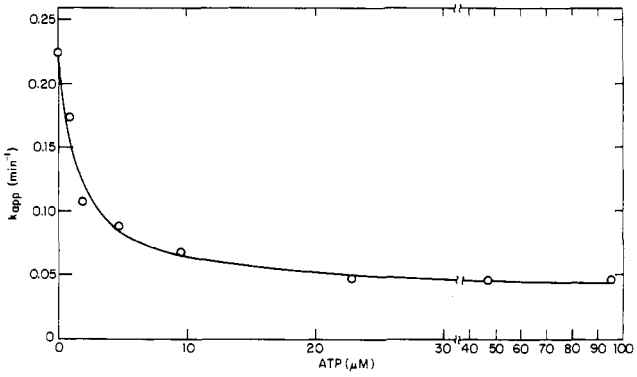

FIGURE 7: A plot of the apparent pseudo-first-order rate constant (k_{app}) for NBD-Cl inhibition of (Na-K)ATPase activity as a function of ATP in the reaction mixture. The reaction conditions were the same as in Figure 5 and a single exponential decay in activity was observed at all ATP concentrations. The protein concentration was 0.2 mg/mL.

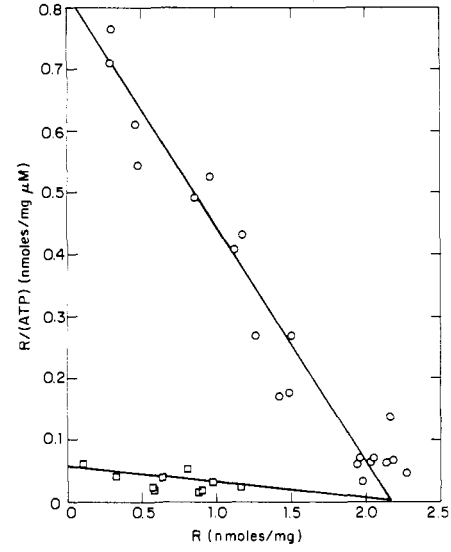

FIGURE 8: The equilibrium binding of [^{3}H]ATP to native enzyme (○) and NBD-reacted-enzyme (□). R is the number of nmoles of ATP bound per mg of protein and (ATP) is the free ATP concentration. The procedure is described in the Experimental Section. The lines were drawn using eq 5 with the parameters n = 2.2 nmol/mg, K_d = 2.6 μM and n = 2.2 nmol/mg, K_d = 50 μM for the native (Na-K)ATPase and NBD-reacted-enzyme respectively.

inactivation data since the absorbance data at long time periods was poorly determined. The best fit values of k_1 and a_{380} were 0.26 min^{-1} and 0.036/(mg mL), respectively. The value of k_1 is in good agreement with the value determined from the PNPase decay (Table I).

Affinity of ATP Site Affording Protection. The affinity of the ATP site affording protection from NBD-Cl inactivation of (Na-K)ATPase activity was determined from the data presented in Figure 7. The pseudo-first-order rate constant for NBD-Cl inactivation was plotted against the ATP concentration in the reaction mixture. Fifty percent maximal protection was observed at 1.5 μM ATP and at saturating ATP (100 μM) the pseudo-first-order rate constant was 0.05 min^{-1}.

The stoichiometry of the ATP site affording protection was determined from the equilibrium binding experiment described in Figure 8. In the absence of NBD-Cl, the data could be described by eq 5

$$R/(\text{ATP}) = n/[(\text{ATP}) + K_d] \quad (5)$$

which assumes one class of ATP binding sites with dissociation constant K_d and stoichiometry n. R is the nanomoles of ATP bound per mg of protein and (ATP) is the free ATP concentration. The values of n and K_d are 2.2 nmol/mg and 2.6 μM, respectively, as determined by a nonlinear least-squares fit of eq 5 to the data in Figure 8. The value of 2.2 nmol/mg is somewhat lower than values of 2.5 to 3.5 nmol/mg determined for the kidney enzyme preparations (Jørgensen, 1974b, Cantley, unpublished results). The dissociation constant (2.6 μM) is in good agreement with the ATP protection constant measured under the same conditions (1.5 μM) showing that this is the site involved in protection against NBD-Cl modification.

The NBD-reacted-enzyme exhibited much weaker ATP binding. Because of the scatter in the data, the value of n (eq 5) could not be accurately determined; however, if n was assumed to be 2.2 nmol/mg, then K was approximately 50 μM indicating a 20-fold lower ATP affinity following NBD-Cl modification.

Discussion

NBD-Cl reacts with two tyrosine residues per (Na-K)-ATPase molecule (6-7 nmol/mg of protein) inhibiting both the ATPase and PNPase activities. A number of cysteine groups are also modified by NBD-Cl; however, a rapid reversal of cysteine modification with 2-mercaptoethanol does not restore ATPase or PNPase activities. The NBD-Cl reacted tyrosine residues undergo a much slower reversal in the presence of 2-mercaptoethanol accompanied by a 65% return of ATPase

TABLE II: NBD-Cl Modification of Various ATPases.

Property	(Na-K)ATPase electroplax	Coupling factors		
		Mito. (F_1)	Chloro. (CF_1)	Bact. (BF_1)
No. of reactive Tyr per enzyme molecule	2	2[a]	2[b]	≤3[c]
Subunit location	95 000	β[d]	β[e]	β[c]
Activity inhibited (%)	ATPase (100%) PNPase (100%)	ATPase (100%)[f] Coupling (100%)[f]	ATPase (100%)[e] -	ATPase (100%)[c,g] Coupling (100%)[g]
No. of Tyr modified for complete ATPase inhibition	1	1[f]	1[b,e]	≤3[c]
Reversibility	Yes (65%)	Yes (100%)[f]	Yes (60%)[h]	Yes (100%)[c,g]
ATP protection	Partial	Partial[f]		
ATP binding site involved in protection (dissociation constant)	Hydrolysis site? (2.6 μM)	Hydrolysis site?[f] (~50 μM)	Hydrolysis site?[i] (7 μM)	- -

[a] Cantley and Hammes, unpublished observations. [b] Cantley and Hammes (1975a). [c] Nelson et al. (1974). [d] Ferguson et al. (1975b). [e] Deters et al. (1975). [f] Ferguson et al. (1975a). [g] Ferguson et al. (1974a). [h] Personal communication from D. Holowka and G. Hammes. [i] Based on NBD-Cl inhibition of AMP-PNP binding (Cantley and Hammes, 1975a).

and PNPase activities. Using [^{14}C]NBD-Cl, the modified tyrosine groups have been located on the 95 000 polypeptide chain of the (Na-K)ATPase. A modification of approximately one tyrosine residue per 95 000 polypeptide chain is sufficient to inhibit the (Na-K)ATPase activity more than 95%.

Masiak and D'Angelo (1975) suggested the location of a tyrosine residue at the active site of the erythrocyte (Na-K)-ATPase on the basis of ATP protected *N*-acetylimidazole inactivation. We were able to reproduce this finding on the eel electroplax enzyme (data not presented) providing additional support for the existence of an essential tyrosine residue.

A model is presented (Figure 6) to explain the effects of Na^+, K^+, and ATP on the inhibition of ATPase and PNPase activities by NBD-Cl. The model suggests two asymmetrically arranged 95 000 polypeptide chains with a single tight ATP binding site. The asymmetry is based on differential reactivity of the two tyrosine groups and partial protection by ATP. The more reactive tyrosine residue has been tentatively located at the tight ATP binding site. This location is determined by three observations: (1) ATP protects this tyrosine group from reaction with NBD-Cl[2]; (2) NBD-Cl modification of this tyrosine reduces the ATP binding affinity; and (3) this tyrosine residue is approximately stoichiometric with the ATP binding capacity and both the ATP binding site and the modified tyrosine residue are located on the 95 000 polypeptide chain.

The role of these tyrosine groups in active Na^+ and K^+ transport is not established. Their reactivity compared with other tyrosine residues in the protein suggests either their phenolic protons exhibit unusually low pK_as or they are readily accessible to the NBD-Cl. Both tyrosine groups are most reactive in the presence of Na^+ (conditions where ATP binds with a high affinity) and less reactive in the presence of K^+ (conditions where ATP binds with a low affinity; Hegyvary and Post, 1971) suggesting a sensitivity to the cation mediated protein conformational state. The model presented implies they are directly involved in ATP or Mg-ATP binding, although an ATP mediated protein conformational change regulating accessibility to these sites cannot be eliminated on the basis of the evidence presented.

The seemingly conflicting evidence between ATP affinities observed under equilibrium and steady-state conditions has raised some debate about the number of ATP sites on the enzyme and their role in ATP hydrolysis. Only a single tight ATP site per 95 000 polypeptide dimer is observed in equilibrium binding experiments presented here in agreement with Jørgensen (1974b); however, under turnover conditions both high affinity and low affinity ATP sites are observed (Cantley and Josephson, 1976; Glynn and Karlish, 1976; Robinson, 1976; Froelich et al., 1976). Using the model in Figure 6 it is tempting to speculate that the less reactive tyrosine residue is the weak site for ATP binding and that ATP must bind to both sites either simultaneously or sequentially during a turnover cycle making modification of either site detrimental to (Na-K)ATPase activity.

The asymmetry in sites presented in this model is compatible with a flip-flop or alternating site mechanism for (Na-K)-ATPase activity as proposed on the basis of steady-state and pre-steady-state kinetics (Schon et al., 1974; Glynn and Karlish, 1976; Froelich et al., 1976; Robinson, 1976; Cantley and Josephson, 1976). In this type of mechanism, the enzyme would flip between energetically equivalent states where Tyr_1 and Tyr_2 are alternately the position of ATP hydrolysis with each turnover. The structural model presented here and the flip-flop mechanism are not unique in explaining the data and merely provide working hypotheses for further investigations.

The potassium stimulated PNPase activity of the (Na-K)ATPase has been investigated in considerable detail. A number of investigators have noted differential inhibition of (Na-K)ATPase and PNPase activities and there have been reports that low concentrations of nucleotides actually stimulate the PNPase activity (for review, see Glynn and Karlish, 1975). Jørgensen (1977) found that tryptic digestions of the rabbit kidney enzyme resulted in differential rates of inhibition of PNPase and (Na-K)ATPase activities. He suggested an asymmetric arrangement of large polypeptide chains with ATPase and PNPase activities on different subunits. Robinson (1976) proposed a similar model on the basis of steady-state kinetics. The model we present here suggests that PNPase activity is occurring at two sites on the enzyme and that ATP will only bind tightly to one of these sites.

Finally, it is interesting to note a number of similarities between the (Na-K)ATPase and the coupling factors of oxidative phosphorylation and photophosphorylation (F_1, BF_1, and CF_1) with respect to inhibition by Nbd-Cl (see Table II). All of these enzymes are inhibited via NBD-Cl modification of an essential tyrosine group. The modified tyrosine residue has been located on the second largest subunit of each coupling factor and is postulated to be at the site for ATP hydrolysis (Cantley and Hammes, 1975a,b). Both the chloroplast coupling factor CF_1 and the mitochondrial coupling factor F_1

[2] Since Na^+ increases the reactivity of this tyrosine group and Na^+ and ATP have been reported to stabilize the same conformational state (Jørgensen, 1975a; Skou, 1974), the protection by ATP is probably due to steric hindrance.

possess two NBD-Cl modifiable tyrosine groups with differential reactivities, only one of which need be modified for inactivation of ATPase activity (Cantley and Hammes, 1975a; unpublished data of Cantley and Hammes). These enzymes have only a single ATP hydrolyzing site measurable by equilibrium binding techniques (Cantley and Hammes, 1975a; Hilborn and Hammes, 1973), although the subunit containing the active site is present as at least a dimer (Baird and Hammes, 1976, 1977). These remarkable similarities in such diverse systems suggest that enzymes coupling ATP hydrolysis to transmembrane cation movement may have similar active sites and mechanisms of action.

Acknowledgment

We thank Professor Guido Guidotti for providing both enthusiasm and financial support throughout this work.

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