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Changes in Chemical Properties of Mitochondrial Adenosinetriphosphatase upon Removal of Tightly Bound Nucleotides†

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ABSTRACT: The removal of tightly bound nucleotides from mitochondrial F_1-ATPase was found to affect the inhibition by ADP and chemical reactivity toward 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) and sulfhydryl reagents. Preincubation of nucleotide-depleted F_1 with 40 μM ADP in the presence of ethylenediaminetetraacetic acid (EDTA) resulted in a 51% inhibition of the steady-state level of ATPase activity whereas only a 25% inhibition was observed for native F_1. Both partially inhibited states of the enzyme could be reversed by the subsequent addition of ATP. Measurement of [^{14}C]ADP binding to nucleotide-depleted F_1 in the presence of EDTA reveals three equivalent ADP binding sites with a K_d of 0.45 μM, and a fourth site of lower affinity. The sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) were found to inhibit the ATPase activity of nucleotide-depleted F_1 but not native F_1 or nucleotide-depleted F_1 in the presence of ADP or ATP. Polyacrylamide gel electrophoresis of nucleotide-depleted F_1 labeled with [^{14}C]NEM gave a 2-fold increase in incorporation into the ($\alpha + \beta$) subunits and a 7-fold increase in label in the γ subunit after 90 min compared to when ADP was present during the reaction. ADP binding to the noncatalytic sites enhanced the rate of inhibition of nucleotide-depleted F_1 by NBD-Cl about 2-fold while retarding the subsequent intramolecular transfer from an essential phenol group to an amino group about 2.8-fold. The results suggest a conformational change in F_1 caused by changes in nucleotide–protein interaction at the noncatalytic sites.

The mitochondrial F_1-ATPase[1] carries the catalytic sites for the synthesis of ATP from ADP and P_i during oxidative phosphorylation. The soluble F_1 portion is composed of five different subunit components (Knowles & Penefsky, 1972a,b), designated α, β, γ, δ, and ϵ. The covalent labeling by photoaffinity analogues of ATP (Wagenvoord et al., 1977; Scheurich et al., 1978; Lunardi & Vignais, 1979; Williams & Coleman, 1982) and phosphate (Lauquin et al., 1980) as well as by alkylating or acylating ATP analogues (Budker et al., 1977; Esch & Allison, 1978; Drusta et al., 1979) has led to the suggestion that the β subunit carries the catalytic site.

Also present on F_1 from mitochondria (Harris et al., 1977; Hashimoto et al., 1981), chloroplasts (Harris & Slater, 1975; Bruist & Hammes, 1981), and bacterial systems (Maeda et al., 1976; Ohta et al., 1980a) are noncatalytic nucleotide binding sites thought to function in regulation. These latter sites have a much higher degree of specificity for adenine nucleotides than the catalytic sites (Harris et al., 1978; Recktenwald & Hess, 1980). Chemical affinity labeling experiments (Kozlov & Milgrom, 1980) in addition to nucleotide binding studies using isolated subunits of *Escherichia coli* (Dunn & Futai, 1980) and thermophilic bacteria (Ohta et al., 1980a) have led to the suggestion that the noncatalytic nucleotide-binding sites are on the α subunits. Nucleotides bound to the noncatalytic sites are empirically termed as "tightly bound" or "nonexchangeable".

More detailed information on the physical and functional relationships between or within the two classes of nucleotide binding sites may be essential to a clear understanding of the mechanisms of synthesis and utilization of ATP by F_1. Cooperativity between multiple hydrolytic sites on isolated F_1 has been demonstrated by Grubmeyer & Penefsky (1981) and Nalin & Cross (1982). In addition, measurements of the rates of the hydrogen exchange on isolated α and β subunits from thermophilic bacteria have provided evidence that the α sub-

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[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; F_1, mitochondrial coupling factor 1; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Hepes–EDTA–glycerol buffer, 50 mM Hepes (pH 7.5), 2 mM EDTA, 25 mM NaCl, and 25% glycerol; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole (also named 4-chloro-7-nitrobenzofurazan); NBD-F_1, F_1 labeled by NBD-Cl; nd-F_1, nucleotide-depleted F_1; NEM, *N*-ethylmaleimide; ATPase, adenosinetriphosphatase.

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units may control the conformation of the β subunits (Ohta et al., 1980b).

In the present work, we have studied the effect of removing tightly bound nucleotides on the chemical properties of soluble F_1. The changes observed in the reactivity of nucleotide-depleted F_1 with sulfhydryl reagents and NBD-Cl likely reflect changes in the conformation mediated by nucleotide–protein interactions.

Experimental Procedures

Materials

ATP, ADP, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, NADH, NADP, hexokinase, glucose-6-phosphate dehydrogenase, 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl), dithiothreitol, 5,5′-dithiobis(2-benzoic acid), and Sephadex G-50-80 were purchased from Sigma Chemical Co. [^{14}C]ADP and [^{14}C]NEM were obtained from New England Nuclear. *N*-Ethylmaleimide and acrylamide were purchased from Aldrich Chemical Co. *N,N,N′,N′*-Tetramethylethylenediamine and ammonium persulfate were purchased from Bio-Rad. PTGC ultrafiltration membranes were supplied by Millipore. All chemicals were of reagent grade quality.

Methods

Preparation of F_1. Mitochondria from beef heart were prepared by the method of Low & Vallin (1963). F_1-ATPase was prepared and stored according to that described by Knowles & Penefsky (1972a). Before use, an aliquot of the ammonium sulfate suspension of F_1 was centrifuged at 10000*g* for 10 min and the pellet dissolved in 50 mM Hepes buffer (pH 7.5) containing 2 mM EDTA, 25 mM NaCl, and 25% glycerol (Hepes–EDTA–glycerol) at a protein concentration of approximately 30 mg/mL. Free and loosely bound nucleotides were removed by desalting the F_1 solution on a 0.9 × 25 cm or 0.5 × 28 cm column of Sephadex G-50 fine in the same buffer. F_1 prepared in this way will be referred to as native F_1.

Nucleotide-depleted F_1 was prepared by the method of Garrett & Penefsky (1975) with a few modifications. An aliquot of an ammonium sulfate suspension of F_1 was centrifuged, and the pellet (usually between 10–20 mg of protein) was dissolved in 1 mL of 100 mM triethanolamine–SO_4 at pH 8.0 containing 4 mM EDTA. The enzyme was immediately precipitated by adding an equal volume of saturated ammonium sulfate solution and placed on ice for 10 min followed by centrifugation to recover the protein pellet. After the precipitation procedure was repeated 2 more times, the pellet was dissolved in 0.3 mL of 100 mM triethanolamine–sulfate, pH 8.0, containing 4 mM EDTA and 50% glycerol. This was applied to a 0.9 × 49 cm column of Sephadex G-50-80 equilibrated with the same buffer. F_1 was eluted approximately 14 h later at a flow rate of 1 mL/h. The fractions containing the highest A_{280}/A_{260} ratios were pooled and reprecipitated by the addition of 1.5 volumes of saturated ammonium sulfate. After 5 min at room temperature, the suspension was centrifuged, and the pellet was dissolved in 0.3 mL of the same buffer containing 50% glycerol. This solution was again applied to the same column after reequilibrating with the 50% glycerol buffer and eluted at the same flow rate as before. No loss in specific activity was observed upon the removal of tightly bound nucleotides from the enzyme. When stored at room temperature in buffers containing 50% glycerol, the specific activity remained stable for at least 2 weeks.

Prior to use, the nucleotide-depleted F_1 stored in 100 mM triethanolamine–sulfate (pH 8.0) containing 4 mM EDTA and 50% glycerol was diluted 2-fold with 50 mM Hepes, pH 7.5, and aliquots were passed through elution–centrifugation columns (Penefsky, 1977) containing Sephadex G-50-80 previously swollen in Hepes–EDTA–glycerol buffer at pH 7.5. After this step, the specific activity decreased about 25% during the first 4 h and remained relatively stable during the next 10 h at room temperature. All experiments with nucleotide-depleted F_1 were started 4 h after the above elution–centrifugation step.

Analysis of Tightly Bound Nucleotides. Tightly bound ATP and ADP were released by incubating F_1 samples with 8 M urea for 20 min on ice. The solutions containing denatured F_1 were applied to a 5 × 11 mm column of 200–400 mesh Dowex 1 anion exchanger (chloride form). The column was then washed with degassed deionized water to remove the urea. Adenine nucleotides were subsequently eluted with 0.01 N HCl and 0.2 M NaCl according to the procedure of Cohn & Carter (1950). The eluted nucleotide peaks were collected while the elution profile was monitored at 259 nm on a Gilson Holochrome. The samples were neutralized by Tris base and immediately frozen at −70 °C. Controls were also run by adding known amounts of ATP or ADP to F_1 samples before or after adding 8 M urea. The recovery was close to 100% in both cases. ATP and ADP were analyzed according to Williamson & Corkey (1969) in coupled enzyme assays utilizing the fluorescence of NADPH and NADH, respectively, on a Hitachi Perkin-Elmer MPF 2A fluorescence spectrophotometer.

Binding Measurements. The binding of [^{14}C]ADP to nucleotide-depleted F_1 was performed by using an ultrafiltration cell designed according to Paulus (1969). The upper and lower chambers were separated by a 13-mm diameter Millipore PTGC disk membrane. Aliquots of enzyme were incubated with different concentrations of [^{14}C]ADP for 1 h before adding to the upper chamber. A pressure of 22 psi of N_2 was applied to the upper chamber, and after 5 min the concentration of unbound [^{14}C]ADP in the lower chamber was measured by adding 10 μL of the filtrate to a scintillation vial containing 5 mL of Aquasol (New England Nuclear). The concentration of bound [^{14}C]ADP was obtained as the difference between the total concentration of [^{14}C]ADP determined before ultrafiltration and the concentration of unbound [^{14}C]ADP in the filtrate.

Assays. ATPase activity was followed by an ATP regenerating system coupled to the oxidation of NADH as previously described (Ting & Wang, 1980). Protein concentration was determined by the Coomassie blue binding method of Bradford (1976) calibrated against either the biuret or Lowry et al. (1951) method. In the latter procedure, bovine serum albumin was used as standard, and the dry weight of F_1 was determined from the correction factor of 1.19 (Kasahara & Penefsky, 1978). Both methods of calibration agreed within experimental error. The molar concentration of F_1 was calculated by using a molecular weight of 360 000 (Lambeth et al., 1971).

The concentrations of ADP and ATP stock solutions were calculated from the absorbance at 259 nm and molar extinction coefficient of 15.4×10^3 M^{-1} cm^{-1} at pH 7.0. Concentrations of NEM stock solutions were determined from the extinction coefficient of 620 M^{-1} cm^{-1} at 350 nm (Riordan & Vallee, 1967).

Chemical Modification by [^{14}C]NEM. In an attempt to saturate nonessential sulfhydryl groups, native F_1 in Hepes–EDTA–glycerol buffer at pH 7.5 was incubated with 1 mM nonradioactive NEM for 3 h at 25 °C. During this time, the

ATPase activity decreased less than 5%. The reaction was terminated by the addition of 1.5 volumes of saturated ammonium sulfate. After 10 min on ice, the suspension was centrifuged, and the supernatant was discarded. The F_1-NEM pellet was dissolved in a small volume of 100 mM triethanolamine–sulfate buffer containing 4 mM EDTA and 50% glycerol. Tightly bound nucleotides were removed as described previously. Prelabeling with NEM did not appear to interfere with the removal of tightly bound nucleotides as evidenced by the A_{280}/A_{260} ratio and the susceptibility to inhibition by sulfhydryl reagents.

Nucleotide-depleted F_1 prepared in this way was incubated with [^{14}C]NEM in Hepes–EDTA–glycerol buffer at pH 7.5. Aliquots were removed at given time intervals for measurement of ATPase activity. At different degrees of inactivation, 0.3-mL samples were freed from unreacted [^{14}C]NEM by elution–centrifugation in 3-mL centrifuge columns. The samples were electrophoresed on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate according to the method of Weber & Osborn (1969). About 250 μg of protein was applied to each 9 cm × 1 cm tube gel and run at 10 mA/gel. The gels were fixed in 12.5% trichloroacetic acid for 4 h and stained overnight with Coomassie blue in 7.5% acetic acid and 20% methanol. The destained gels were sliced in 2 mm thick sections and placed in glass scintillation vials. Each gel slice was macerated with a glass stirring rod and digested with 0.5 mL of 30% H_2O_2 for 20 h at 60 °C in a shaking water bath. At this stage 10 mL of Aquasol was added to each vial for counting of radioactivity. No loss in the ^{14}C radioactivity was observed after the control nonradioactive gel slices were digested in the presence of known quantities of [^{14}C]NEM.

Inhibition of ATPase Activity by NBD-Cl. Rates of inactivation of F_1-ATPase activity were initiated by the addition of 1 μL of an ethanolic stock solution of NBD-Cl to 100 μL of enzyme in Hepes–EDTA–glycerol buffer. The reaction mixture was incubated at 25 or 30 °C in the dark with aliquots removed at time intervals for ATPase measurements. The controls were incubated as above in the absence of NBD-Cl but with the addition of 1 μL of ethanol.

Measurement of the Rate of NBD Transfer from Tyrosine to a Neighboring Amino Group and Hydrolysis Reactions. Nucleotide-depleted F_1 at 1.97 mg/mL was preincubated with 42 μM ADP in Hepes–EDTA–glycerol buffer for 2 h before inactivating with NBD-Cl until 3% ATPase activity remained. The reaction was stopped by removing unreacted NBD-Cl as well as separating free and loosely bound ADP from the enzyme by elution–centrifugation in the same buffer not lacking ADP and NBD-Cl. The centrifugation was started immediately (usually less than 10 s) after applying the enzyme to the centrifuge column. At this stage, a control sample that was preincubated with 40 μM [^{14}C]ADP contained 2.7 mol of [^{14}C]ADP/mol of enzyme. Another sample of nucleotide-depleted F_1 under the same conditions but lacking exogenous ADP was inactivated by NBD-Cl until 5% ATPase activity remained. The NBD-F_1 and NBD-ADP-F_1 eluted from the centrifuge columns were incubated in the dark at 25 °C. Samples were taken at time intervals and assayed for ATPase activity with and without 2.5 mM DTT in the assay mixture. Controls were subject to the same treatment as their corresponding samples except for the inactivation step by NBD-Cl.

Results

Analysis of Tightly Bound Nucleotides. In view of the variation of the reported values (Garrett & Penefsky, 1975; Leimgruber & Senior, 1976; Harris et al., 1977; Wagenvoord et al., 1980) it was of interest to determine independently the content of tightly bound ATP and ADP of native F_1 under our conditions. The results presented in Table I show that native F_1 with an A_{280}/A_{260} ratio of 1.51 contains about 1.38 mol of ADP/mol of F_1 and 1.39 mol of ATP/mol of F_1 of M_r 360000. Since these nucleotides remained tightly associated with F_1 after either repeated ammonium sulfate precipitations or gel filtration, they are consistent with the operationally defined term of "tightly bound nucleotides" described by Harris (1978). After prolonged standing at room temperature (72 h) in Hepes–EDTA–glycerol buffer at pH 7.5 followed by nucleotide analysis, 1.89 mol of ADP/mol of F_1 and 0.68 mol of ATP/mol of F_1 were recovered. This increase in the ADP content is approximately equal to the decrease in ATP recovered. The specific activity decreased by about 50% during the 72-h period. This suggests that storage conditions and/or time of assay may be a factor in the variability of the reported value.

Table I: Stoichiometry of Tightly Bound Adenine Nucleotides in F_1-ATPase from Beef Heart Mitochondria

F_1 sample	A_{280}/A_{260}	ADP/F_1 (mol/mol)	ATP/F_1 (mol/mol)	(ADP+ATP)/F_1 (mol/mol)
native F_1[a]	1.51	1.38 ± 0.05	1.39 ± 0.07	2.77 ± 0.06
native F_1[b]	1.51	1.89 ± 0.04	0.68 ± 0.09	2.57 ± 0.08
"native" F_1[c]	1.86	0.79 ± 0.05	0.05 ± 0.01	0.84 ± 0.04
nd-F_1[d]	2.05	0.07 ± 0.02	0.04 ± 0.01	0.11 ± 0.02

[a] Native F_1 freshly prepared by desalting the enzyme on a column of Sephadex G-50 fine in Hepes–EDTA–glycerol buffer to remove free and rapidly exchanging nucleotides. [b] Native F_1 aged 72 h at room temperature in Hepes–EDTA–glycerol buffer. [c] F_1 precipitated 3 times by ammonium sulfate followed by passage through the first 50% glycerol column. [d] F_1 after second 50% glycerol column. Details for the preparation of native and nucleotide-depleted F_1 are described under Methods. Nucleotides were extracted and analyzed according to that described under Methods. The values listed include the standard deviation. A_{280}/A_{260} represents the absorbance at 280 nm divided by the absorbance at 260 nm.

Our method of preparing nucleotide-depleted F_1 required an additional passage through the column of Sephadex G-50-80 to fully deplete all tightly bound nucleotides. After a single passage through the column, F_1 (A_{280}/A_{260} = 1.89) was essentially depleted of bound ATP but still retained about 0.79 mol of ADP/mol of F_1. However, a second passage through the column removed the remaining bound ADP from the enzyme giving an A_{280}/A_{260} greater than 2.

Binding of [^{14}C]ADP to Nucleotide-Depleted F_1. The capacity for nucleotide-depleted F_1 to bind [^{14}C]ADP in the presence of EDTA at pH 7.5 is shown in Figure 1. The Scatchard type plot indicates approximately three equivalent ADP binding sites with a dissociation constant of 0.45 μM. At higher ADP concentrations a fourth molecule of ADP binds similar to that observed by Garrett & Penefsky (1975).

Effect of Preincubation with Nucleotides on ATPase and ITPase Activity. The different degrees of inhibition of ATPase activity obtained by preincubating native vs. nucleotide-depleted F_1 with 40 μM ADP is shown in Figure 2. In this experiment, aliquots of the enzyme ADP mixtures in Hepes–EDTA–glycerol buffer were removed at the indicated time intervals and diluted about 500-fold in the ATPase assay medium. Despite the large dilution factor and the presence of pyruvate kinase and phosphoenolpyruvate to convert any free ADP to ATP during the ATPase measurements, an inhibited steady-state level of ATP hydrolysis was obtained. It is shown that within 4 min of preincubation time with 40 μM ADP, the steady-state level of ATP hydrolysis reached a maximum of 51% inhibition for nucleotide-depleted F_1 while

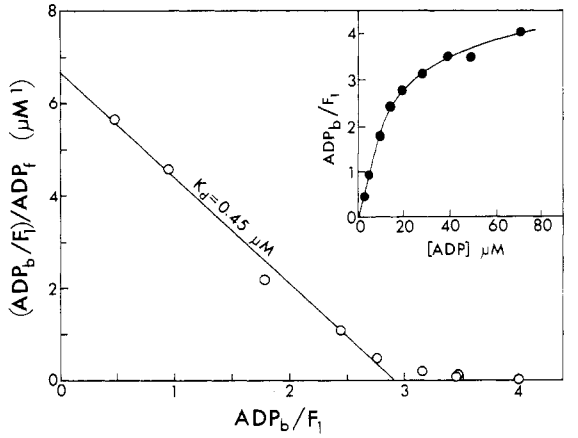

FIGURE 1: Binding of [^{14}C]ADP to nucleotide-depleted F_1. Enzyme samples at 1.89 mg/mL were incubated at room temperature in Hepes–EDTA–glycerol buffer containing the indicated concentrations of [^{14}C]ADP (8.9×10^4 cpm/nmol) shown in the inset. After 1 h, 0.2-mL aliquots were transferred to the ultrafiltration cell for measurement of binding (see Methods). The data shown in the inset were subsequently plotted by the method of Scatchard.

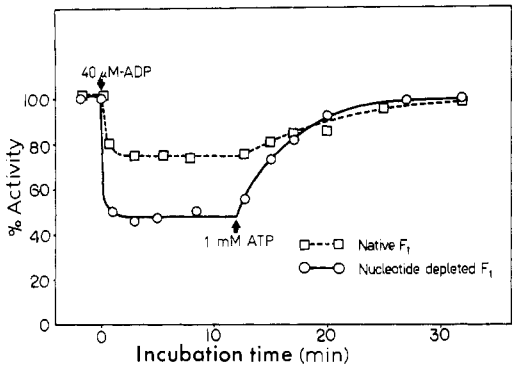

FIGURE 2: Time course of ADP-induced inhibition of ATPase activity of native and nucleotide-depleted F_1 and subsequent reversal by ATP. At zero time 40 μM ADP was added to 1.6 mg/mL native or nucleotide-depleted F_1 in Hepes–EDTA–glycerol buffer. Aliquots were withdrawn at intervals and assayed for ATPase activity as described under Methods. Reversal of inhibition was initiated by adding 1 mM ATP to the F_1 samples at 12-min incubation time and measured by assaying the ATPase activity as a function of time; 100% activity corresponds to a specific activity of 62 units/mg for native F_1 and 47 units/mg for nucleotide-depleted F_1.

only a 25% inhibition was observed for native F_1. The addition of 1 mM ATP to the enzyme–ADP samples after 12 min leads to a time dependent reversal of the inhibited state. Harris et al. (1978) have previously shown that tightly bound nucleotides become more exchangeable with nucleotides in the medium in the presence of EDTA or upon brief exposure to low pH. From their study, it was concluded that the replacement of tightly bound ATP by ADP can lead to partial inhibition of the ATPase activity. Our results can be explained similarly. Since native F_1 used in our experiments contains about 1.38 mol of ADP/mol of F_1 and 1.39 mol of ATP/mol of F_1, the lower degree of ADP-induced inhibition compared to nucleotide-depleted F_1 may be due to the failure of 40 μM ADP to displace the last tightly bound ATP. Nucleotide-depleted F_1 may be inhibited to a greater degree by ADP because of the direct binding of ADP to the unoccupied high-affinity sites. No inhibition of the ATPase activity was observed when nucleotide-depleted F_1 was preincubated with 2.4 mM IDP (not shown). Furthermore, preincubation of nucleotide depleted F_1 with 60 μM ADP can lead to 63% inhibition of ITPase activity when 2.5 mM ITP replaced the ATP in the assay

Table II: Effect of Various Compounds on the Rate of Inactivation by DTNB[a]

enzyme and experimental conditions	[DTNB] (mM)	% activity after 45 min
nd-F_1	0.4	61
nd-F_1 + 25 μM ATP	0.4	100
nd-F_1 + 29 μM ADP	0.4	100
native F_1	0.4	100
nd-F_1	1.3	54
nd-F_1 + 5 mM phosphate	1.3	94
nd-F_1 + 20 mM sulfate	1.3	100
nd-F_1-ADP[b]	1.3	99

[a] F_1 samples in Hepes-EDTA-glycerol buffer were preincubated with the indicated reagents for 1 h at 25 °C before the addition of DTNB. After 45 min, the ATPase activity was determined for each sample. Controls without DTNB lost no ATPase activity during this time. Fresh stock solutions of phosphate and sulfate were prepared from their sodium salts and adjusted to pH 7.5 before adding to the F_1 samples. [b] nd-F_1 (nucleotide-depleted F_1) incubated with 26 μM ADP in the presence of 1.5 mM $MgCl_2$ for 30 min followed by elution-centrifugation in Hepes-EDTA-glycerol buffer. Controls treated in this way using [^{14}C]ADP contained approximately 3.1 mol of [^{14}C]ADP/mol of F_1.

mixture. In both cases, the enzyme samples were diluted 500-fold in the assay mixture. These results are consistent with the higher degree of specificity of the noncatalytic sites for adenine nucleotides (Harris et al., 1978).

Inhibition of Nucleotide-Depleted F_1 by DTNB. The effects of DTNB on the ATPase activity of various F_1 samples are summarized in Table II. Nucleotide-depleted F_1 in Hepes–EDTA–glycerol buffer was inhibited approximately 39% by 0.4 mM DTNB after 45 min. In contrast, no inhibition by DTNB was observed with native F_1 or nucleotide-depleted F_1 preincubated with 25 μM ATP or 29 μM ADP. Both 20 mM sulfate and 5 mM phosphate were able to protect against inactivation by 1.3 mM DTNB (100% and 94% ATPase activity, respectively, remained after 45 min). The similarity in their protective effects is not surprising in view of the structural similarity of the two anions. Last, preincubating nucleotide-depleted F_1 with 26 μM ADP in the presence of $MgCl_2$, followed by elution–centrifugation in Hepes–EDTA–glycerol buffer to remove free and loosely bound nucleotide as well as the Mg^{2+} ions, prevented subsequent inactivation by DTNB. This result taken together with the lack of inhibition observed with native F_1 strongly suggests that the protection against inactivation afforded by nucleotides is due to binding to the noncatalytic high-affinity sites.

Prolonged incubation of nucleotide-depleted F_1 with DTNB and NEM can eventually lead to nearly 100% inhibition. However, long incubation times resulted in protein aggregates that were not seen in native or nucleotide-reconstituted F_1 samples under the same condition.

Time Course of [^{14}C]NEM Incorporation into F_1 Subunits. The effect of removing tightly bound nucleotides on the rate of labeling by [^{14}C]NEM is illustrated in Figure 3. The increased labeling of nucleotide-depleted F_1 after 90 min relative to the enzyme preincubated with ADP can be attributed to a 2-fold increase in [^{14}C]NEM incorporation into the ($\alpha + \beta$) subunits and a 7-fold increase in label associated with the γ subunit. The δ and ϵ subunits in both enzyme samples after this time contained less than 0.008 mol of [^{14}C]NEM/mol of enzyme and, therefore, are not included in the figure. Due to the large quantity of protein applied to the gels for more reliable counting of radioactivity and the similarity in molecular weights, the α and β subunits were not clearly separated on the gels. Hence, reference has been made to ($\alpha + \beta$). When smaller quantities of protein are applied

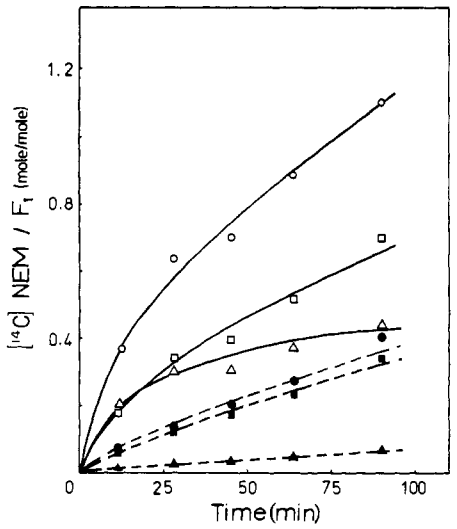

FIGURE 3: Time course of [^{14}C]NEM incorporation into F_1 subunits. Nucleotide-depleted F_1 was prepared as described under Methods. The enzyme sample at 2.23 mg/mL in Hepes–EDTA–glycerol buffer was divided into two portions: one incubated with 49 μM ADP for 1 h and the other without ADP. [^{14}C]NEM (1.82×10^4 cpm/nmol) was added to each enzyme sample at time zero, and 0.3-mL aliquots at given time intervals were freed from unreacted [^{14}C]NEM by elution–centrifugation. The samples were subsequently run on 10% polyacrylamide gels in the presence of $NaDodSO_4$. The bands visualized by Coomassie blue staining were sliced and digested for radioactive counting as described under Methods. The [^{14}C]NEM incorporation into nd-F_1 (○, □, △) and the ADP-preincubated enzyme (●, ■, ▲) are shown. (○, ●) Total incorporation; (□, ■) ($\alpha + \beta$) band; (△, ▲) γ subunit. No significant radioactivity was found in the δ and ϵ subunits. The ATPase activity of the nd-F_1 sample was inhibited 62% while the ADP-preincubated F_1 lost no ATPase activity after 90-min incubation with [^{14}C]NEM.

to $NaDodSO_4$–polyacrylamide gels for better separation of these two subunits, approximately 75–80% of the radioactivity in the ($\alpha + \beta$) band was found to be in the α subunit of both enzyme samples after 90-min incubation with [^{14}C]NEM. The finding of [^{14}C]NEM label in the β subunit was confirmed when the enzyme samples were run on urea gels according to the method of Knowles & Penefsky (1972a). This is not in accord with an earlier report demonstrating the absence of cysteine in the β subunit of beef heart F_1 (Senior, 1975). However, amino acid analysis of "subunit 2" (Knowles & Penefsky, 1972b) and the finding of [^{14}C]NEM label associated with the band corresponding to the β subunit in urea gels (Lauquin et al., 1980) indicates that the β subunit may in fact contain cysteine. Nucleotide-depleted F_1 contained about 1.1 mol of [^{14}C]NEM/mol of enzyme after 90 min, resulting in approximately 60% inhibition of ATPase activity. Because the increase in [^{14}C]NEM labeling of nucleotide-depleted F_1 is not restricted to an enhanced incorporation into any one subunit, it is difficult to determine the location of the sulfhydryl groups responsible for the inhibition of the ATPase activity. Since the enzyme preincubated with ADP still contained [^{14}C]NEM label in the ($\alpha + \beta$) and γ subunits (but to a much lower extent) without loss of ATPase activity, it appears that these labeled sulfhydryl groups are nonessential for ATPase activity.

Inhibition of ATPase Activity by NBD-Cl. NBD-Cl has been shown to be a potent inhibitor of native F_1 upon reacting with an essential tyrosine residue (Ferguson et al., 1975a), probably in one of the β subunits (Lunardi & Vignais, 1979). Figure 4 shows that the time course for the inactivation of both native and nucleotide-depleted F_1 follows pseudo-first-order kinetics. However, nucleotide-depleted F_1 is clearly inactivated at a slower rate than the native enzyme containing about three

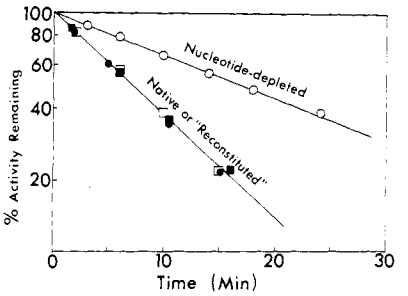

FIGURE 4: Inactivation of ATPase activity by NBD-Cl. Native F_1 and nd-F_1 were prepared as described under Methods. The reaction conditions were the following: Hepes–EDTA–glycerol buffer, pH 7.5, 25 °C, 49 μM NBD-Cl, 0.8 mg/mL native F_1 (●), and 1.0 mg/mL nd-F_1 (○); (■, □) native or nd-F_1, respectively, preincubated with 29 μM ADP for 1 h before the addition of NBD-Cl. At the indicated time intervals, aliquots were withdrawn for measurement of ATPase activity as described under Methods.

tightly bound nucleotides. A sample of the enzyme was first preincubated with 29 μM ADP prior to the reaction with NBD-Cl in order to test whether the different rate of inhibition of nucleotide-depleted F_1 is due to possible damage during its preparation. This concentration of ADP was sufficient to allow binding to the three high-affinity sites (see Figure 1, inset). The results demonstrate that the addition of ADP enhanced the rate of inactivation to a value equal to that of native F_1. When the same concentration of ADP was incubated with native F_1, no effect on the inactivation rate was observed.

Effect of Tightly Bound ADP on the Kinetics of the Intramolecular NBD Transfer and Hydrolysis Reactions. Once reacted with an essential tyrosine residue, the NBD label can slowly transfer to an amino group in the γ subunit (O → N transfer) and thereby becomes stable even in the presence of DTT (Ferguson et al., 1975b). Alternatively, a gradual loss of NBD label can occur from the enzyme which is accompanied by an increase in ATPase activity (Ferguson et al., 1975b; Lunardi et al., 1979), presumably due to hydrolysis of the NBD group from the tyrosine residue. The effects of ADP bound to the noncatalytic sites on the rate of the NBD transfer and hydrolysis reactions are presented in Figure 5. In this experiment, NBD-labeled, ADP-reconstituted F_1 containing about 2.7 mol of ADP/mol of F_1 and NBD-labeled, nucleotide-depleted F_1 were assayed for ATPase activity at time intervals with (circles) or without (squares) DTT in the assay medium. Initially, about 100% of the ATPase activity was recovered by DTT in the ADP-reconstituted enzyme (Figure 5A), suggesting all the NBD label was on the essential tyrosine residue. With DTT present during the assay, a time-dependent decrease in the specific activity was observed due to the O → N transfer reaction, while the hydrolysis reaction is evidenced by the slow increase in specific activity in the absence of DTT. In comparison, the data for the NBD-labeled, nucleotide-depleted F_1 sample shown in Figure 5B appear quite different. Initially, only 65% of the ATPase activity can be recovered by DTT relative to the control. This observation seems to indicate that some of the NBD levels on nucleotide-depleted F_1 may have been due to the rapid direct labeling of an essential amino group(s). A functional lysine residue at the catalytic site has previously been implicated (Ting & Wang, 1981; Koga & Cross, 1982).

The data in Figure 5 may be treated quantitatively. Let us adopt the following notation: C, C', and C'' represent the molar concentrations of unlabeled F_1, O-NBD-labeled F_1, and N-NBD-labeled F_1, respectively, at time t after excess NBD-Cl has been removed; C_0, C_0', C_0'' represent the corresponding concentrations at $t = 0$; k_1 and k_2 represent the first-order rate

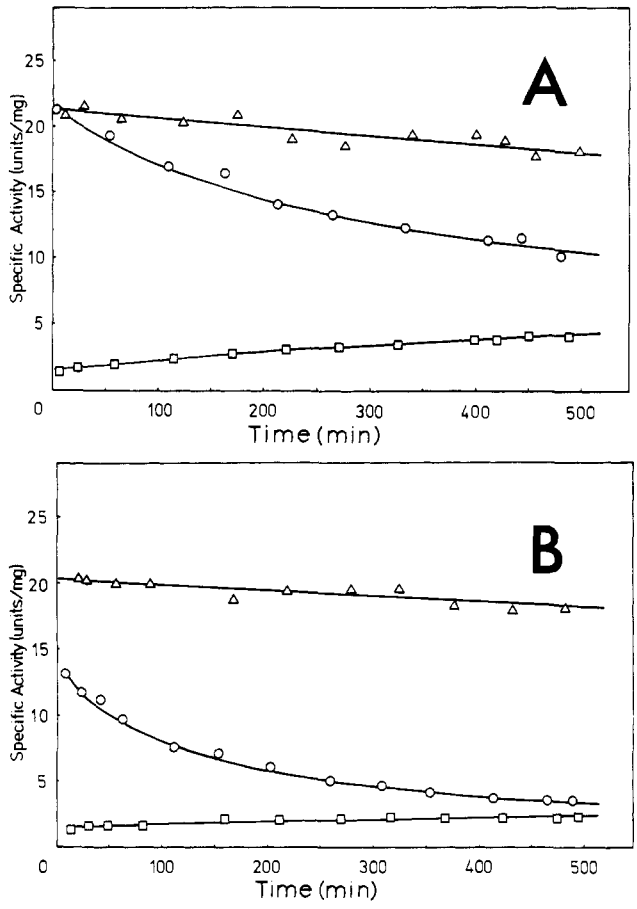

FIGURE 5: Measurement of ATPase activity as a function of time of NBD-modified F_1 samples which were assayed in the presence and absence of DTT. F_1 samples were modified by NBD-Cl as described under Methods. Time zero was defined as that at the start of the elution–centrifugation procedure which removed the unreacted NBD-Cl. (A) ADP-reconstituted F_1: NBD-labeled ADP-F_1 was incubated in the dark at 25 °C, and aliquots were removed at time intervals for ATPase activity measurements in the presence (○) or absence (□) of 2.5 mM DTT. From the cpm of controls, the ADP-reconstituted F_1 after elution–centrifugation contained approximately 2.7 mol of [^{14}C]ADP/mol of F_1. (B) Nucleotide-depleted F_1: The conditions for the NBD-labeled nucleotide-depleted F_1 were the same as those in (A). ATPase activity was measured in the presence (○) or absence (□) of 2.5 mM DTT. Unmodified controls in both (A) and (B) are represented by (△). The specific activity of the controls were not changed by 2.5 mM DTT in the assay.

constants of the NBD-transfer and NBD-hydrolysis reactions, respectively. For the activity recoverable by DTT, we have

$$-\frac{dC'}{dt} = (k_1 + k_2)C' \quad (1)$$

which upon integration gives

$$\ln C' = -(k_1 + k_2)t + \ln C_0' \quad (2)$$

or

$$C' = C_0' \exp[-(k_1 + k_2)t] \quad (3)$$

Plotting the data according to eq 2 gives a slope of $k' = k_1 + k_2 = 0.278$ h^{-1} for NBD-labeled, nucleotide-depleted F_1 and $k' = 0.125$ h^{-1} for NBD-labeled, ADP-reconstituted F_1.

For the O → N transfer reaction, we have

$$\frac{dC''}{dt} = k_2C' = k_2C_0' \exp[-(k_1 + k_2)t] \quad (4)$$

Integration gives

$$\frac{C'' - C_0''}{C_0'} = \frac{k_1}{k_1 + k_2}[1 - e^{-(k_1+k_2)t}] \quad (5)$$

Similarly

$$\frac{C - C_0}{C_0'} = \frac{k_2}{k_1 + k_2}[1 - e^{-(k_1+k_2)t}] \quad (6)$$

In the case of NBD-labeled, ADP-reconstituted F_1, the data in Figure 5A when calculated according to eq 5 and 6 gives $k_1 = 0.092 \pm 0.006$ h^{-1} and $k_2 = 0.032 \pm 0.002$ h^{-1}. Similarly, the data in Figure 5B for the NBD-labeled, nucleotide-depleted F_1 show that $k_1 = 0.259 \pm 0.008$ h^{-1} and $k_2 = 0.027 \pm 0.004$ h^{-1}. Therefore, we may conclude that the NBD-transfer reaction is much faster in nucleotide-depleted F_1 than in ADP-reconstituted F_1.

Discussion

In this report, the removal of tightly bound nucleotides from soluble mitochondrial F_1-ATPase is shown to change its reactivity toward sulfhydryl reagents and NBD-Cl. Both DTNB and NEM can inhibit the ATPase activity of nucleotide-depleted F_1 but not native F_1 or nucleotide-depleted F_1 reconstituted with nucleotides. Since native F_1 contains about three adenine nucleotides which are tightly bound even in the absence of Mg^{2+}, it appears that the occupation of these sites by ADP or ATP can prevent the inhibition of ATPase activity by the sulfhydryl reagents. This may account for the lack of inhibition of soluble F_1 noted by others (Pullman et al., 1960; Senior, 1973; Godinot et al., 1977) since native F_1 was used in their studies.

Interestingly, Di Pietro et al. (1980) reported that the ATPase activity of nucleotide-depleted F_1 from pig heart was not inhibited by 6.7 mM *p*-(chloromercuri)benzoate. The apparent discrepancy between their results and ours may be due to the presence of sulfate in their buffers. Indeed, we found that sulfate and phosphate have a protective effect against inactivation by 1.3 mM DTNB.

The enhanced rate of [^{14}C]NEM labeling of the ($\alpha + \beta$) and γ subunits of nucleotide-depleted F_1 compared to the rate when ADP was present during the reaction implies that either the bound nucleotides can sterically hinder the reactive sulfhydryl group at the nucleotide binding site or that their removal from the enzyme can induce a conformational change that exposes the normally unreactive sulfhydryl groups. Since only the α and β subunits have been shown to bind nucleotides, the 7-fold increase in [^{14}C]NEM bound to the γ subunit after 90 min compared to the γ subunit from the enzyme preincubated with ADP allows us to favor the latter possibility.

The results from the rates of inhibition by NBD-Cl and subsequent intramolecular transfer to an amino group is also consistent with a different conformation in F_1 when tightly bound nucleotides are removed. The increase in the second-order rate constant of inhibition by NBD-Cl after preincubating with ADP was found to occur with nucleotide-depleted F_1, but not native F_1, suggesing that the noncatalytic sites are involved. Ting & Wang (1980) have previously observed the lack of effect of ADP binding to the catalytic site on the inactivation rate by NBD-Cl under similar conditions. As expected, the rebinding of ADP by nucleotide-depleted F_1 as the three high affinity sites ($K_d = 0.45$ μM in the absence of Mg^{2+}) decreases the rate constant for the O → N transfer of the NBD label from 0.259 ± 0.008 h^{-1} to 0.092 ± 0.006 h^{-1}.

The location of the essential phenol group labeled by NBD-Cl is not completely settled. It was suggested previously that NBD-Cl may not react at the catalytic site (Ferguson et al., 1976; Esch & Allison, 1979). This view is not inconsistent with the lack of effect of NBD-Cl modification on the stoichiometry of AMP-PNP binding to the exchangeable sites observed by Cross & Nalin (1982). However, their obser-

vations that the K_d of the first AMP-PNP bound to NBD-F_1, which probably carried only one NBD label per F_1 molecule, is more than 5 times as large as the corresponding value for native F_1 whereas the K_d values for the second and third AMP-PNP bound to NBD-F_1 are equal to those for native F_1 strongly supports the suggested location of the NBD label at or near the catalytic site. In addition, the protective effect of P_i, ATP, and ATP analogues on the rate of inactivation by NBD-Cl (Ting & Wang, 1980), and the finding that prior modification prevents the photolabeling of the β subunit by aryl azide derivatives of ADP and ATP (Lunardi & Vignais, 1979) also suggest that this tyrosyl residue may be at or near a nucleotide binding site on the β subunit. In light of the evidence mentioned in the introduction that the noncatalytic sites are on the α subunits while the catalytic sites are on the β subunits, it seems likely that the binding of ADP to the α subunits can influence the conformation of the β subunits. A similar suggestion has previously been made by Ohta et al. (1980b) based on the kinetics of hydrogen-exchange reactions of the isolated α and β subunits from thermophilic bacteria. A regulatory function for the noncatalytic sites was proposed since these investigators found that in the presence of ATP, the α subunit tightened the structure of the β subunit.

In the present study, the inhibitory effect on the ATPase activity by tightly bound ADP and the ability of ATP to reverse the partially inhibited state would also be suggestive of a regulatory role for the noncatalytic sites. If the noncatalytic sites do indeed serve a regulatory role, their possible effect on the interaction between subunits as well as the cooperative interactions between the catalytic sites (Nalin & Cross, 1982; Cross et al., 1982; Gresser et al., 1982) seems to imply a very complex mechanism of action for this coupling factor in oxidative phosphorylation.

Acknowledgments

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Registry No. ATPase, 9000-83-3; ATP, 56-65-5; ADP, 58-64-0; NBD-Cl, 10199-89-0; DTNB, 69-78-3; NEM, 128-53-0.

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Mechanism of Glutamate Transport in *Escherichia coli* B. 1. Proton-Dependent and Sodium Ion Dependent Binding of Glutamate to a Glutamate Carrier in the Cytoplasmic Membrane†

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ABSTRACT: Specific binding of glutamate to its carrier was investigated by using cytoplasmic membrane vesicles prepared from *Escherichia coli* B. The binding activity was specifically affected by the Na^+ and H^+ concentrations of the medium. Cytoplasmic membrane vesicles from the mutant strain 36-39 that is defective in the Na^+-dependent glutamate transport system showed no binding of glutamate. Addition of the protonophore uncoupler 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile or carbonyl cyanide *m*-chlorophenylhydrazone, or the ionophore monensin or nigericin, did not inhibit the binding, indicating that the binding reaction is not energy dependent. The parameters of binding were determined in reaction media with various combinations of H^+ and Na^+ concentrations. The maximum number of binding sites was constant and determined to be 70 pmol/mg of membrane protein, irrespective of the concentrations of H^+ and Na^+ in the medium. The apparent dissociation constant, however, was greatly affected by changes in the concentrations of both H^+ and Na^+, in such a way that it was expressed by a linear combination of the reciprocals of the H^+ and Na^+ concentrations. The characteristics of binding can be explained best by supposing that glutamate can bind only to a H^+/Na^+/carrier complex that is formed by random binding of H^+ and Na^+ to the unloaded carrier. The physiological role of this elementary binding reaction and of this quaternary complex as an active intermediate in the process of glutamate transport is discussed.

Studies on the energetics of active solute transport in bacteria have progressed extensively since proposition of the chemiosmotic hypothesis (Mitchell, 1966, 1967; Rosen & Kashket, 1978). Carrier proteins are recognized to function mostly as secondary chemiosmotic pumps that utilize an electrochemical gradient of proton or sodium ion as a driving force. However, the molecular mechanism of active transport catalyzed by syn-coupled and anti-coupled reactions remains unsolved (Mitchell, 1967; Rosen & Kashket, 1978).

Frank & Hopkins (1969) demonstrated that glutamate transport in *Escherichia coli* B and its derivatives is stimulated by Na^+ and that this stimulation is due to an increase in the affinity for substrate. Glutamate transport activity was retained on membrane vesicles, and a periplasmic binding protein was not intimately involved in the transport process (Minor & Frank, 1974). Later, two groups of investigators proposed the mechanism of a Na^+/glutamate symport (Hasan & Tsuchiya, 1977; Tsuchiya et al., 1977; MacDonald et al., 1977), based on the observations that a chemical gradient of Na^+ imposed directly on intact cells and membrane vesicles caused the accumulation of glutamate.

Our current interest in active transport has been focused specifically on the initial step of the H^+/substrate symport reaction. Thus, we have investigated the specific binding of proline to its carrier in membrane vesicles of *Escherichia coli* and the effects of H^+ and/or Na^+ on the binding (Amanuma et al., 1977; Anraku, 1982; Motojima et al., 1979; T. Mogi and Y. Anraku, unpublished results). The results of these studies and of the present work indicated that co-ions (H^+ and Na^+) initially bind to a carrier that catalyzes a syn-coupled reaction and that the binary or ternary complex of the carrier with co-ions then binds substrate to form an intermediate for translocation across the cytoplasmic membrane.

In this paper, we demonstrate the specific stimulatory effect of H^+, in addition to Na^+, on glutamate binding and propose a model of glutamate binding to the carrier. In the following paper (Fujimura et al., 1983), we propose a model of glutamate transport based on the binding model and describe the mechanism of syn-coupled transport of $Na^+/H^+/Glu^-$ via the glutamate carrier.

Experimental Procedures

Bacterial Strains. Escherichia coli B (wild strain) and its derivative, strain 36-39, were obtained from T. Tsuchiya (Okayama University, Okayama). Strain 36-39 is resistant to methyl DL-α-glutamate and shows low activity of Na^+-independent glutamate uptake (Tsuchiya et al., 1977). These strains were grown in Na^+-free medium B7 (Frank & Hop-

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