duction of substrate 6dEb during development of the mycelium? The C-6 erythranolide hydroxylase may play an important regulatory role in the biosynthesis of the erythromycins, and continued study of this enzyme system may thus be important for a variety of reasons.

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Functional Groups at the Catalytic Site of BF_1 Adenosinetriphosphatase from *Escherichia coli*†

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ABSTRACT: The rates of inactivation of BF_1 adenosinetriphosphatase (BF_1-ATPase) from *Escherichia coli* by 7-chloro-4-nitro-2,1,3-benzoxadiazole, 1-fluoro-2,4-dinitrobenzene, 2,4,6-trinitrobenzenesulfonate, phenylglyoxal, and *N,N'*-dicyclohexylcarbodiimide have been measured in the presence and absence of various concentrations of inorganic phosphate, ADP, ATP, or magnesium ion. Dissociation equilibrium constants and rate constants for the labeling reactions have been deduced from a quantitative treatment of the kinetic data. The results suggest that the essential Tyr, Lys, Arg, and Glu or Asp residues are probably located at the catalytic site of BF_1-ATPase and that in addition to steric interference, the effect of charge interaction should also be considered in interpreting the kinetic data on the protection of BF_1-ATPase by substrate molecules against inactivation by the above labeling reagents. Examination of the relative values of the rate constants for the labeling reactions in the presence and absence of inorganic phosphate, ADP, ATP, or magnesium ion, respectively, and the effect of NBD label on the rates of labeling of the essential guanidinium, amino, and carboxyl groups suggest that the arrangement of these four functional groups at the catalytic site of BF_1 may be similar to that previously proposed for MF_1-ATPase from bovine heart; namely, the essential amino group and the unusually reactive phenol group are probably located near the bound inorganic phosphate or the γ-phosphate group of the bound ATP, the essential guanidinium group is probably located nearer to the α- or β-phosphate group than to the γ-phosphate group of the bound ATP or the bound inorganic phosphate, and the essential carboxylate group is probably complexed with a magnesium ion which it shares with the bound inorganic phosphate.

Escherichia coli BF_1-ATPase[1] has the subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Bragg & Hou, 1975; Yoshida et al., 1979) or $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$ (Vogel & Steinhart, 1976). The isolated enzyme has 3 mol of tightly bound nucleotides, 2 mol of ATP, and 1 mol of ADP per mol of enzyme (Maeda et al., 1976). The tight nucleotide sites seem to be on the α subunits, since the isolated α subunit contains a single tight nucleotide binding site with a K_d value of 0.1 μM for ATP and 0.9 μM for ADP, whereas the β, γ, δ, and ϵ subunits do not bind ATP or ADP in the ligand concentration range from 0.1 to 2 μM (Dunn and Futai, 1980). One of the tight nucleotides may be more rapidly exchangeable with external nucleotides with a K_d value of 1 μM for ADP (Lunardi et al., 1981). *E. coli* BF_1-ATPase has three loose nucleotide binding sites with a K_m of 200–400 μM for ATP hydrolysis (Futai et al., 1974), the K_i for ADP inhibition in hydrolysis is ~80 μM, and K_d for ADP at the

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EEDQ, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; FDNB, 1-fluoro-2,4-dinitrobenzene; FSBA, 5'-[(*p*-fluorosulfonyl)benzoyl]adenosine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; NAP_4-ADP, 3'-*O*-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]adenosine 5'-diphosphate; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole (also named 4-chloro-7-nitrobenzofurazan); PEP, phosphoenolpyruvate; PG, phenylglyoxal; TNBS, 2,4,6-trinitrobenzenesulfonate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; BF_1, *Escherichia coli* coupling factor; MF_1, beef heart mitochondrial coupling factor; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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low-affinity binding site is 50 μM (Lunardi et al., 1981). Dicyclohexylcarbodiimide (DCCD) has been shown to react with one carboxyl group on the β subunit of *E. coli* BF_1-ATPase to fully inactivate the enzyme (Satre et al., 1979). 7-Chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) reacts with a very reactive phenol group of Tyr on the β subunit to inactivate the enzyme (Lunardi et al., 1979). Previous labeling of BF_1-ATPase with NBD-Cl did not affect the binding of 3'-*O*-(4-[*N*-(4-azido-2-nitrophenyl)amino]butyl)adenosine 5'-diphosphate (NAP_4-ADP) to the exchangeable tight site on the α subunit, but significantly decreased the loose nucleotide binding on the β subunit, which suggests that the Tyr which is particularly reactive toward NBD-Cl is at the catalytic site of *E. coli* BF_1-ATPase (Lunardi et al., 1981).

Phenylglyoxal has been used to modify the essential Arg residue in mitochondrial MF_1-ATPase (Marcus et al., 1976; Frigeri et al., 1977, 1978; Kohlbrenner & Cross, 1978, 1979; Ting & Wang, 1980a), chloroplast CF_1-ATPase (Andreo & Vallejos, 1977), and *Rhodospirillum rubrum* BF_1 (Vallejos et al., 1978). Fluorodinitrobenzene (FDNB) probably reacts with Tyr on MF_1-ATPase, since the labeling can be partially reversed by 90 mM DTT (Andrews & Allison, 1981). 2,4,6-Trinitrobenzenesulfonate (TNBS) was found to inactivate MF_1-ATPase through covalent labeling with the amino group which was not reversed by 90 mM DTT (Ting & Wang, 1981).

In the present work, *E. coli* BF_1-ATPase was inactivated by NBD-Cl, DCCD, PG, FDNB, and TNBS. The effect of binding of P_i, Mg^{2+}, ADP, and ATP on the inactivation rate by these reagents was investigated. The kinetically deduced dissociation equilibrium constants indicate that the effect results from the binding of ligands at the catalytic site of BF_1-ATPase. These results suggest that Tyr, Lys, Asp or Glu, and Arg are functional amino acid residues at the catalytic site which are probably involved in the ATP hydrolysis and synthesis as suggested for MF_1 (Ting & Wang, 1980b).

Experimental Procedures

Materials. Pyruvate kinase (Type II), L-lactate dehydrogenase (Type III), phosphoenolpyruvate, ATP (equine muscle; crystalline), ADP (Grade I), NADH (Grade III), NBD-Cl, FDNB, TNBS, phenylglyoxal, and DTT were purchased from Sigma Chemical Co. DCCD was purchased from Aldrich Chemical Co. Sephadex G-50 fine was the product of Pharmacia Fine Chemicals, Inc. All other chemicals used were of reagent grade. Frozen cells of *E. coli* K12 grown in a medium containing yeast extract, glucose, and salts and harvested in the late exponential phase were purchased from Miles Biochemicals.

Preparation of E. coli BF_1-ATPase. Frozen cells of *E. coli* K12 bought from Miles Biochemicals were washed and resuspended in 50 mM Mops–KOH, pH 7.0, 10 mM $MgCl_2$, and 2.5 mM 2-mercaptoethanol. Membranes were prepared by disruption of the cells in a French Press at 18 000 psi of pressure of 10–15 °C. *E. coli* BF_1-ATPases were released from the membranes by washing membranes with low ionic strength buffer and purified by poly(ethylene glycol) fractionations and gel filtration (Vogel, 1979). Purified *E. coli* BF_1-ATPase was stored in 50 mM Mops buffer, pH 7.9, 2.5 mM 2-mercaptoethanol, and 20% methanol at 4 °C. The specific activity was 25 units/mg of protein at 30 °C. The purified *E. coli* BF_1 has a molecular weight of 350 000 (Paradies & Schmidt, 1979) and is composed of five subunits, designated α, β, γ, δ, and ε, with molecular weights of 58 000, 52 000, 31 000, 20 000, and 13 000, respectively, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The *E. coli* BF_1-ATPase was desalted in a 1 × 30 cm Sephadex G-50 fine column, with a buffer containing 50 mM Hepes, pH 8.0, 25 mM NaCl, 2 mM EDTA, and 25% glycerol.

ATPase Assay. ATPase activity was assayed in 2 mL of a solution containing 50 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 5 mM ATP, 1.5 mM PEP, 0.2 mM NADH, 90 units of pyruvate kinase, and 18 units of L-lactic dehydrogenase at pH 8.0 and 30 °C. The absorbance change at 340 nm was followed in a Gilford Model 250 spectrophotometer.

Protein Concentration. The protein concentration was determined by the Bio-Rad protein assay with Coomassie blue G-250 as described by Bradford (1976). Bovine γ-globulin from Bio-Rad was used as a standard.

Inactivation of E. coli BF_1-ATPase by NBD-Cl, FDNB, TNBS, Phenylglyoxal, and DCCD. In each inactivation experiment of *E. coli* BF_1-ATPase by NBD-Cl, FDNB, TNBS, and DCCD, respectively, 2 μL of a methanolic stock solution of the reagent was added to 200 μL of a BF_1-ATPase in 50 mM Hepes, pH 8.0 or 7.0, 25 mM NaCl, 2 mM EDTA, and 25% glycerol. The mixture was then incubated at 30 °C in the dark, and aliquots were taken out at intervals for ATPase assay. In the control experiment, 2 μL of methanol was added to 200 μL of the BF_1-ATPase solution. For the inactivation study of BF_1-ATPase by phenylglyoxal, 10 μL of an aqueous stock solution was added to 200 μL of the BF_1-ATPase solution. In the control experiment, 10 μL of distilled H_2O was added. In the experiments for studying the effect of P_i, ATP, ADP, or Mg^{2+} on the inactivation rate of BF_1-ATPase by NBD-Cl, FDNB, TNBS, PG, or DCCD, the BF_1-ATPase solution was preincubated with the particular ligand for 2 h at room temperature.

Effect of NBD Labeling on the Inactivation of E. coli BF_1-ATPase by FDNB, TNBS, PG, and DCCD. A solution of BF_1-ATPase was divided into two portions. To one portion was added NBD-Cl in methanol to a final concentration of 100 μM. To the second portion, used as the control, was added an equivalent volume as methanol. After 30 min at 24 °C in the dark, the portion with NBD-Cl lost 95% of its original ATPase activity. The two samples were subsequently freed from the excess of NBD-Cl by elution–centrifugation (Penefsky, 1977). Both the BF_1-ATPase control and NBD–BF_1 samples were then mixed with the second labeling reagent and incubated at 30 °C. Aliquots of both mixtures were then taken out at intervals and assayed for ATPase activity in the medium containing 2 mM DTT. The DTT was used to remove the NBD label from Tyr and to restore the lost ATPase activity due to NBD labeling.

Results

Ligand Effect on the Inactivation Rate of E. coli BF_1-ATPase by NBD-Cl. At any given concentration of NBD-Cl, the decay of ATPase activity followed first-order kinetics, indicating the inactivation rate is first order with respect to $[BF_1]$. The linear plot of log $t_{1/2}$ against log [NBD-Cl] over the range investigated gives a slope of −0.83 which indicates that the rate of inactivation is also first order with respect to [NBD-Cl]. Consequently, the rate of inactivation may be written as

$$-\frac{d[F_1]}{dt} = k'[F_1][R] \qquad (1)$$

where R represents the labeling reagent. At 2 mM, DTT could remove the NBD label from NBD–BF_1 and restore the ATPase activity within 20 s almost completely. The observed continued decrease in the inactivation rate as the concentration of P_i is raised as shown in Figure 1 indicates that P_i can protect

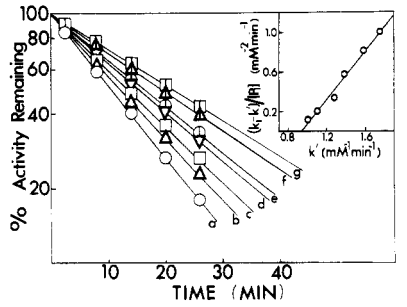

FIGURE 1: Effect of different concentrations of P_i on the inactivation of BF_1-ATPase by NBD-Cl. 1.14 μM BF_1 in 50 mM Hepes, 25 mM NaCl, 2 mM EDTA, and 25% glycerol, pH 8.0, was preincubated with different concentrations of P_i at 24 °C for 2 h and then inactivated by 32.9 μM NBD-Cl at 30 °C in the dark. Concentrations of P_i are (a) 0, (b) 0.28, (c) 0.57, (d) 1.14, (e) 2.27, (f) 4.55, and (g) 9.09 mM. The inset shows the determination of the dissociation constant (K_P) of P_i bound at this active site in BF_1.

BF_1 against inactivation by NBD-Cl. At any given concentration of the excess reagent, the observed rate of inactivation by NBD-Cl is equal to the sum of the labeling rates of free BF_1 and of F_1 with bound P_i, respectively, i.e.

$$-\frac{d[F_1]}{dt} = (k_1[F_1]_{free} + k_2[F_1 \cdot P_i])[R] \quad (2)$$

and hence the familiar expression

$$\frac{1}{[R]}\left(-\frac{d \ln [F_1]}{dt}\right) = k_1\left(\frac{1}{1 + [P_i]/K_P}\right) + k_2\left(\frac{[P_i]/K_P}{1 + [P_i]/K_P}\right) \quad (3)$$

where K_P represents the dissociation constant for P_i bound to this NBD-Cl reactive site on BF_1 and k_1 and k_2 represent the apparent rate constants for the inactivation reaction without P_i and with $[P_i] \gg K_P$, respectively. Integration of eq 3 gives

$$\ln [F_1]/[F_1]_{total} = -k'[R]t \quad (4)$$

where the apparent rate constant k' is given by

$$k' = k_1\left(\frac{K_P}{K_P + [P_i]}\right) + k_2\left(\frac{[P_i]}{K_P + [P_i]}\right) \quad (5)$$

which, upon rearrangement, becomes

$$(k_1 - k')/[P_i] = (k' - k_2)/K_P \quad (6)$$

Since k_1 is determined directly from the observed inactivation rate in the control experiment in the absence of P_i, the values of K_P and k_2 can be readily obtained from the slope and intercept of the linear plot of the observed values of $(k_1 - k')/[P_i]$ vs. k' at constant [NBD-Cl]. Such a linear plot is shown in the inset of Figure 1. The values so determined for the labeling of F_1 by NBD-Cl are k_1 = 2.04 mM^{-1} min^{-1}, k_2 = 0.93 mM^{-1} min^{-1}, and K_P = 0.80 mM at 30 °C in Hepes buffer containing 25 mM NaCl and 2 mM EDTA at pH 8.0.

Table I shows that Mg^{2+} alone does not protect BF_1-ATPase against inactivation by NBD-Cl, but Mg^{2+} can increase the P_i protective effect. At fixed Mg^{2+} concentration (2.7 mM), the dissociation constant of the bound P_i is about 0.20 mM and k_2 is approximately 0.43 mM^{-1} min^{-1} at 30 °C in the same buffer. Thus, the observed dissociation constant of the bound P_i in the presence of Mg^{2+} (K_{MgP}) is only one-fourth of its value in the absence of Mg^{2+} (K_P), and the k_2 value in the presence of Mg^{2+} is about half of its value in the absence of Mg^{2+}. It has been shown that Mg^{2+} also helps the P_i binding and that K_{MgP} is only one-third of K_P in MF_1-ATPase (Kasahara & Penefsky, 1978; Ting & Wang, 1980b).

Table I: Effect of Phosphate, Adenine Nucleotides, and Mg^{2+} on the Inactivation of BF_1-ATPase by NBD-Cl[a]

experiment	$t_{1/2}$ (min)
control	10.3
+4.55 mM ATP	10.3
+4.55 mM ADP	6.6
+4.55 mM P_i	19.3
+4.55 mM Mg^{2+}	10.3
+0.28 mM P_i	12.0
+0.28 mM P_i + 2.70 mM Mg^{2+}	21.2
+0.57 mM P_i	13.3
+0.57 mM P_i + 2.70 mM Mg^{2+}	26.0
+1.14 mM P_i	15.2
+1.14 mM P_i + 2.70 mM Mg^{2+}	33.2

[a] Experimental conditions were the same as those for Figure 1.

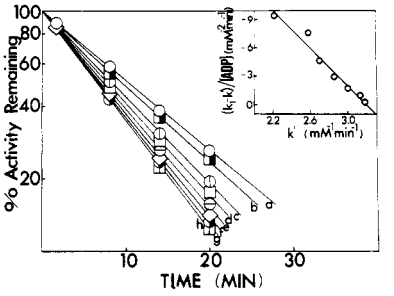

FIGURE 2: Effect of different concentrations of ADP on the inactivation of BF_1 by NBD-Cl. The reaction conditions were the same as in Figure 1. Concentrations of ADP are (a) 0 mM, (b) 17.8 μM, (c) 71 μM, (d) 142 μM, (e) 284 μM, (f) 568 μM, (g) 1.14 mM, and (h) 4.55 mM. The inset shows the determination of the dissociation constant (K_{ADP}) of ADP bound to BF_1.

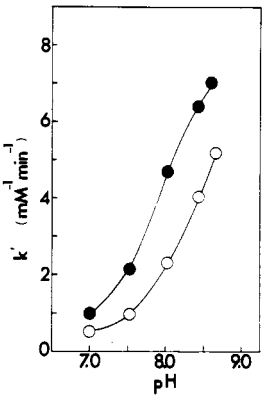

FIGURE 3: Effect of pH on the inactivation of BF_1 (○) and ADP-preincubated BF_1 (●) by NBD-Cl. ADP-preincubated BF_1 was prepared by the preincubation of BF_1 with 6.7 mM ADP for 2 h. The concentration of ADP used is much higher than K_d at any pH. 1.14 μM BF_1 or ADP-preincubated BF_1 was inactivated at 30 °C in 50 mM Hepes buffer containing 25 mM NaCl, 2 mM EDTA and 25% glycerol at the indicated pH.

The results in Table I also show that ATP does not affect the inactivation rate of BF_1-ATPase by NBD-Cl. On the other hand, ADP increases the inactivation rate. The effect of different concentrations of ADP on the inactivation rate of BF_1 by excess NBD-Cl was studied to find the cause of the activation as shown in Figure 2. The data when treated similarly gives k_1 = 2.04 mM^{-1} min^{-1}, k_2 = 3.20 mM^{-1} min^{-1}, and K_{ADP} = 100 μM at 30 °C in 50 mM Hepes buffer containing 25 mM NaCl and 2 mM EDTA at pH 8.0. This result seems to suggest that the activation effect of ADP does not result from the binding of ADP to the tight nucleotide site(s), since the K_d value of the tight binding site for ADP is around 1 μM (Dunn & Fatai, 1980; Lunardi et al., 1981). Figure 3 shows the effect of pH on the inactivation of BF_1 and BF_1

Table II: Effect of Phosphate, Adenine Nucleotides, and Mg^{2+} on the Inactivation of BF_1-ATPase by FDNB[a] and PG[b]

reagent	experiment	$t_{1/2}$ (min)
FDNB	control	8.5
FDNB	+4.55 mM Mg^{2+}	8.5
FDNB	+4.55 mM ADP	10.7
FDNB	+4.55 mM ATP	13.7
FDNB	+4.55 mM P_i	16.0
PG	control	6.8
PG	+4.55 mM Mg^{2+}	6.8
PG	+4.55 mM ADP	17.9
PG	+4.55 mM ATP	21.5
PG	+4.55 mM P_i	10.2

[a] Experimental conditions were the same as those for Figure 4. [b] Experimental conditions were the same as those for Figure 1, except 3.9 mM PG was used as the labeling reagent.

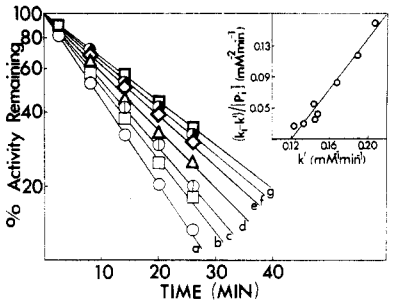

FIGURE 4: Effect of different concentrations of P_i on the inactivation of BF_1-ATPase by FDNB. The reaction conditions were the same as in Figure 1 except FDNB (329 μM) was used instead of NBD-Cl. Concentrations of P_i are (a) 0, (b) 0.227, (c) 0.455, (d) 0.909, (e) 2.27, (f) 3.64, and (g) 4.55 mM. In the inset, there are two extra points calculated from data of $[P_i]$ = 1.82 mM and $[P_i]$ = 2.73 mM, respectively.

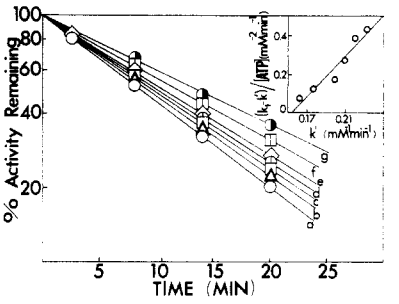

FIGURE 5: Effect of different concentrations of ATP on the inactivation of BF_1-ATPase by FDNB. The reaction conditions were the same as in Figure 1. Concentrations of ATP are (a) 0 mM, (b) 35.5 μM, (c) 71 μM, (d) 142 μM, (e) 284 μM, (f) 568 μM, and (g) 1.14 mM.

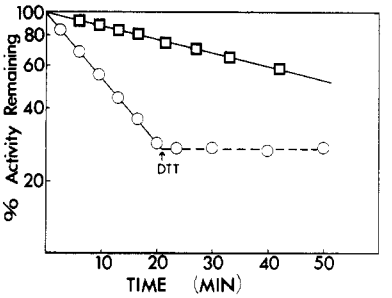

FIGURE 6: Inactivation of BF_1-ATPase by TNBS. 1.14 μM BF_1-ATPase in 50 mM Hepes, pH 8.0, 25 mM NaCl, 2 mM EDTA, and 25% glycerol was inactivated by 0.33 mM TNBS at 30 °C in the dark (○). DTT (final concentration 91 mM) was added at the time indicated by the arrow. In the experiment on the protective effect of P_i, BF_1 was preincubated with 16.9 mM P_i in the same buffer at 24 °C and subsequently inactivated by 0.33 mM TNBS at 30 °C in the dark (□).

preincubated with ADP at a concentration $\gg K_d$. The results indicate a decrease in the apparent pK_a of the reaction center caused by the bound ADP at the catalytic site, since the K_{ADP} value is consistent with K_i and K_d values for ADP bound at the catalytic site of BF_1-ATPase (Lunardi et al., 1981). In the case of MF_1-ATPase, ADP does not change the inactivation rate of MF_1 by NBD-Cl at pH 8.0 whereas ATP does protect MF_1 against the inactivation by NBD-Cl. It has also been observed that MF_1 preincubated with ADP or ATP at a concentration $\gg K_d$ has but a slightly decreased apparent pK_a (unpublished data).

Ligand Effect on the Inactivation Rate of E. coli BF_1-ATPase by FDNB. BF_1-ATPase could be inactivated by an excess of FDNB. The inactivation reaction is first order with respect to either $[BF_1]$ or [FDNB]. Most of the ATPase activity can be restored by adding 90 mM DTT for 30 min. This result suggests that the amino acid residue which is labeled by FDNB with resultant loss of ATPase activity is probably a Tyr residue as shown in MF_1 (Andrews & Allison, 1981).

Table II demonstrates that P_i or ATP effectively protects and ADP slightly protects, whereas Mg^{2+} by itself does not protect *E. coli* BF_1-ATPase against inactivation by FDNB. Figures 4 and 5 show the effect of different concentrations of P_i and ATP on the inactivation of BF_1 by FDNB. Data from Figure 4 give the value of k_1 = 0.243 mM^{-1} min^{-1}, k_2 = 0.115 mM^{-1}, and K_P = 0.65 mM. Results from Figure 5 give the value of k_1 = 0.247 mM^{-1} min^{-1}, k_2 = 0.154 mM^{-1} min^{-1}, and K_{ATP} = 190 μM. The value of K_{ATP} is consistent with the K_M value for ATP at the catalytic site of BF_1-ATPase (Futai et al., 1974). The dissociation constant K_P is also decreased by the presence of Mg^{2+}. These results are consistent with the results on MF_1-ATPase (Ting & Wang, 1980b). Since FDNB may modify the same Tyr as NBD-Cl does, it is surprising that ADP enhances NBD-Cl inactivation but slightly protects BF_1-ATPase against FDNB inactivation; ATP has no effect on NBD-Cl inactivation but protects against FDNB inactivation.

Inactivation of BF_1-ATPase by TNBS. The amino reagent TNBS was found to inactivate MF_1-ATPase (Ting & Wang, 1981). It was also found to inactivate BF_1-ATPase, and the inactivation was not reversed by 90 mM DTT as shown in Figure 6. This observation excludes the possibility that the inactivation of BF_1-ATPase is through the modification of Tyr by TNBS. The negative charge of TNBS gives it an affinity to the active site. In the experiment on the protective effect of P_i on the inactivation of BF_1-ATPase by TNBS, the concentration of TNBS used was much lower than K_M, and the concentration of P_i was also quite high. Figure 6 shows that P_i effectively protects BF_1-ATPase from inactivation by TNBS. The same result has been demonstrated in MF_1-ATPase (Ting & Wang, 1981). The observed protection of BF_1 and MF_1 by P_i against TNBS suggests that the essential amino group labeled by TNBS is probably at the catalytic site.

Inactivation of BF_1-ATPase by PG. BF_1-ATPase was found to be inactivated by PG (Table II). The inactivation is first order with respect to either $[BF_1]$ or [PG], since the linear plot of log $t_{1/2}$ against log [PG] gives a slope of −0.95. Table II shows that ATP protects more than ADP does and P_i slightly protects, whereas Mg^{2+} alone does not protect BF_1-ATPase against inactivation by PG. This observation is very similar to that on MF_1-ATPase (Ting & Wang, 1980a).

Inactivation of BF_1-ATPase by DCCD. Previous work showed that 1 mol of DCCD reacts with an essential carboxyl group in a β subunit per mol of BF_1-ATPase to inactivate the enzyme (Satre et al., 1979). The effects of P_i, ATP, ADP,

Table III: Effect of Phosphate, Adenine Nucleotides, and Mg^{2+} on the Inactivation of BF_1-ATPase by DCCD

	experiment	$t_{1/2}$ (min)
BF_1:[a]	control	4.0
	+4.55 mM Mg^{2+}	32.0
	+4.55 mM ADP	4.0
	+4.55 mM ATP	7.9
	+4.55 mM P_i	7.8
BF_1:[b]	control	13.9
	+2.0 mM EDTA	9.9
	+4.55 mM Mg^{2+}	72.0

[a] BF_1 in 50 mM Hepes, pH 7.1, 25 mM NaCl, 2 mM EDTA, and 25% glycerol was inactivated by 30.6 μM DCCD at 30.5 °C in the dark. [b] BF_1 in 50 mM Hepes, pH 7.0, 25 mM NaCl, and 25% glycerol was inactivated by 10.3 μM DCCD at 30.5 °C in the dark.

and Mg^{2+} on BF_1-ATPase in pH 7.1 buffer containing 2 mM EDTA are summarized in Table III. ADP did not change the $t_{1/2}$ of inactivation. Either ATP or P_i increased the $t_{1/2}$ by a factor of 2. Mg^{2+} can by itself protect BF_1 against inactivation by DCCD effectively. Satre et al. (1979) reported that $t_{1/2}$ was increased by a factor of 2 in the presence of 10 mM ATP or 10 mM ADP in pH 6.5 MOPS buffer without EDTA.

Table III shows that preincubation of BF_1 in Hepes buffer, pH 7.1, with 2 mM EDTA decreases the $t_{1/2}$ of inactivation by DCCD. Such an observation seems to suggest that there is endogenous Mg^{2+} in BF_1 in the buffer without EDTA which can increase the $t_{1/2}$ of DCCD inactivation. This phenomenon was also observed in the inactivation of MF_1 in the same buffer by EEDQ. The addition of exogenous Mg^{2+} to BF_1 in the buffer without EDTA was shown to increase the $t_{1/2}$ of DCCD inactivation further. On the other hand, exogenous Mg^{2+} did not change the $t_{1/2}$ of EEDQ inactivation of MF_1 in the same buffer (Ting & Wang, 1980b).

Effect of NBD Label on the Inactivation of BF_1-ATPase by FDNB, PG, DCCD, and TNBS. Since all these reagents inactivate BF_1-ATPase via covalent labeling of BF_1 at the catalytic site, it would be of interest to know whether modification by one reagent affects the subsequent modification by another. Since 2 mM DTT in the ATPase assay mixture can remove the NBD label on the active Tyr in BF_1, the enzyme was first inactivated to zero activity by NBD-Cl and subsequently labeled by other reagents. The inactivation rate of the second reagent was followed by taking out aliquots of the incubation mixture at different time intervals and assayed for ATPase activity in the presence of 2 mM DTT.

As shown in Table IV, NBD labeling did not change the $t_{1/2}$ of PG inactivation of BF_1. For MF_1, NBD labeling was found to facilitate the PG inactivation. FDNB inactivation on BF_1 was slowed down by NBD labeling; the reason may be because both FDNB and NBD-Cl label the same Tyr at the catalytic site. The $t_{1/2}$ of DCCD inactivation on BF_1 was decreased by NBD-Cl labeling. But EEDQ inactivation on MF_1 was not affected by NBD-Cl labeling. There is only a slight difference in the $t_{1/2}$ of TNBS inactivation on BF_1 and NBD–BF_1. By contrast, NBD labeling seems to decrease $t_{1/2}$ of TNBS inactivation on MF_1.

Table IV: Effect of NBD Label on the Inactivation of BF_1-ATPase by PG, FDNB, DCCD, and TNBS

	$t_{1/2}$ (min)	
reagent	BF_1[a]	NBD-BF_1[b]
PG	12.0	11.8
FDNB	4.8	13.7
DCCD	32.2	17.0
TNBS	10.3	12.0

[a] BF_1 in 50 mM Hepes, pH 8.0, 25 mM NaCl, 2 mM EDTA, and 25% glycerol was inactivated by 1.61 mM PG, 0.49 mM FDNB, 30 μM DCCD, or 0.32 mM TNBS. The ATPase assays were done in the presence of 2 mM DTT. [b] NBD-F_1 in the same buffer was prepared as described under Experimental Procedures. The ATPase assays were made in the presence of 2 mM DTT.

Discussion

It is generally believed that the ADP and ATP tightly bound to the α subunits of F_1-ATPases affect the activity of these enzymes and hence may have regulatory roles. For example, preincubation of the native MF_1-ATPase as prepared from bovine heart with ADP could decrease the ATPase activity almost to half of its original value. In order to avoid the unnecessary complication due to possible slow exchange of the tightly bound nucleotides during the labeling reaction, all BF_1 samples for substrate protection studies were preincubated with the corresponding concentrations of ADP or ATP for 2 h before the labeling reagent was added.

When the ATPase activity was monitored as a function of preincubation time with ADP or ATP in a series of preliminary experiments, it was found that preincubation for 2 h was sufficient for most of the samples to reach exchange equilibrium between free nucleotides in solution and the nucleotides bound to the high-affinity sites. To be consistent, the samples with P_i or Mg^{2+} were also preincubated for 2 h before the labeling reagents were added. The observed concentration dependence of the protection of BF_1 by ADP or ATP against the labeling reagents should be due essentially to the nucleotides bound at the catalytic sites, not at the high-affinity sites. This is because the experimental plots gave K_{ADP} and K_{ATP} values which are very close to the corresponding K_d values for the catalytic sites which are often larger by a factor of 10^2 than those for the high affinity sites.

The inactivation of *E. coli* BF_1-ATPase by NBD-Cl, FDNB, TNBS, PG, and DCCD suggests that certain Tyr, Lys, Arg, and Glu or Asp residues are essential for the activity of this ATPase. The results on the effects of P_i, ADP, ATP, or Mg^{2+} on the inactivation of BF_1-ATPase by these reagents are summarized in Table V. The same set of essential amino acid residues have been found in MF_1-ATPase by chemical modification studies (Ting & Wang, 1980a,b, 1981). Data on the effect of P_i, ADP, ATP, or Mg^{2+} on the inactivation of MF_1 by these reagents are also included in Table V for comparison. The kinetically determined equilibrium dissociation constants of P_i, ADP, and ATP confirm that the observed effect of these ligands on the rate of covalent labeling is caused by the binding of these ligands at the catalytic site, not at the tight binding site(s). The values of k_1 and k_2 for each labeling reagent represent the inactivation rate constants of ATPase alone and ATPase bound with a particular ligand, respectively. It is important to note that in most of the cases the value of k_2 is not zero, which suggests that these ligands cannot completely prevent the labeling reaction of either BF_1-ATPase or MF_1-ATPase. Consequently, at high ligand concentration, the inactivated BF_1 or MF_1 may have the same ligand to protein stoichiometry as the corresponding native enzyme, even though some of the ligand dissociation equilibrium constants may be quite different from those of the native enzyme.

The chemical inactivation by group-specific reagents and the observed effect of P_i, ADP, ATP, or Mg^{2+} bound at the catalytic site on the rates of inactivation of BF_1 and MF_1 (Table V) do not necessarily imply that the essential phenol, amino, guanidinium, and carboxyl groups are at the catalytic site. One could assume that none of them is at the catalytic

Table V: Summary of Kinetic and Dissociation Constants for BF_1 and MF_1

	NBD-Cl		FDNB		TNBS		PG		DCCD,	EEDQ,
	BF_1	MF_1	BF_1	MF_1	BF_1	MF_1	BF_1	MF_1	BF_1	MF_1
K_P (mM)	0.80	1.3	0.65	1.1				1.4		
K_{MgP} (mM)	~0.20	0.43								
K_{ADP} (μM)	100	210						160		
K_{ATP} (μM)			190							
K_{Mg} (μM)										8.9
k_1 (mM^{-1} min^{-1})	2.04	3.5	0.25	0.11	0.19	0.087	0.026	0.022	5.66	0.072
$k_2(F_1 \cdot P_i)$ (mM^{-1} min^{-1})	0.93	0.27	0.12	0.048	0.04	0.071		0.011		0.07
$k_2(F_1 \cdot MgP_i)$ (mM^{-1} min^{-1})	0.43[a]	~0								0.032[a]
$k_2(F_1 \cdot ADP)$ (mM^{-1} min^{-1})	3.20	3.5[a]	0.20[a]	0.088[a]			0.010[a]	0.005	5.66[a]	0.07[a]
$k_2(F_1 \cdot ATP)$ (mM^{-1} min^{-1})	2.04[a]	1.8[a]	0.15	0.06[a]			0.008[a]	0.005[a]	2.87[a]	0.07[a]
$k_2(F_1 \cdot Mg)$ (mM^{-1} min^{-1})	2.04[a]	3.5[a]	0.25[a]	0.11[a]			0.026[a]	0.022[a]	0.71[a]	0.032

[a] The approximate rate constant was determined by one measurement at [ligand >> K_D rather than from a linear plot of many measured values over a range of ligand concentrations as shown in Figure 1.

site but that the covalent labeling of each triggers a long-range conformational change which inactivates the ATPase and that the binding of P_i, ADP, ATP, or Mg^{2+}, respectively, at the catalytic site likewise triggers different degrees of long-range conformational change which could change the inactivation rate constant from k_1 to k_2. Such a hypothetical case cannot be ruled out, but the following observations suggest that the essential phenol, amino, guanidinium, and carboxyl groups are not far from each other and most probably are at the catalytic site of both MF_1-ATPase and BF_1-ATPase. (1) The NBD label on the Tyr transfers spontaneously to an Lys residue at alkaline pH and the *N*-NBD-labeled ATPase is also inactive in both BF_1 and MF_1 (Ferguson et al., 1975a,b; Lunardi et al., 1979). (2) The NBD label on the Tyr enhances the rate of labeling of the essential Arg by the hydrophobic reagent phenylglyoxal in MF_1 (Ting & Wang, 1980a). (3) The NBD label on Tyr enhances the rate of labeling of the essential Lys residue by the hydrophobic anionic reagent TNBS in MF_1 (Ting & Wang, 1981). (4) The NBD label on Tyr enhances the rate of labeling of the essential carboxyl group by DCCD in BF_1 (Table IV). (5) Mg^{2+} protects MF_1 and BF_1 against inactivation by EEDQ and DCCD, respectively, presumably due to its complexation with the essential carboxyl group (Table V). (6) Mg^{2+} helps the binding of P_i to MF_1 (Kasahara & Penefsky, 1978) and also enhances the protection of MF_1 and BF_1 by low concentrations of P_i against inactivation by NBD-Cl and FDNB. These observations suggest that the Mg^{2+} ion may be ligated by both P_i and the essential carboxyl group so that it assists in the binding of P_i (Table V). (7) NBD–BF_1 decreased the binding of photoactive reagents to the loose nucleotide binding sites on β subunits (Lunardi et al., 1981). (8) P_i or ATP protects BF_1 from inactivation by DCCD (Table III).

Table V shows that the relative values of k_1 and k_2 for the inactivation of BF_1 by most of the listed reagents are consistent with those in MF_1, except that while ADP has no effect on the rate of inactivation of MF_1 by NBD-Cl, it enhances the rate of inactivation of BF_1 and that while ATP retards the inactivation of MF_1 by NBD-Cl, it has no effect on the rate of inactivation of BF_1 at pH 8.0. The pH dependence of the NBD-Cl reaction is shown in Figure 3 which suggests that the enhanced rate of inactivation of BF_1 by NBD-Cl in the presence of ADP may be due to a decrease in the apparent pK_a of the reaction center caused by the ADP bound at the catalytic site of BF_1. Similar pH-dependence studies showed that the apparent pK_a of the reaction center of MF_1 is decreased only slightly by ADP. This observation seems to indicate that both steric hindrance and electrostatic interaction due to the bound ligand at the catalytic site could affect the rate of inactivation of the ATPase by NBD-Cl. Stereochemical interference tends to make $k_2/k_1 < 1$, whereas the negative charge of the ligand tends to make $k_2/k_1 > 1$ by pulling the proton away from the essential Tyr so that a greater percentage of its functional group can exist in the more reactive phenolate form.

It is possible that the above two effects happen to compensate each other in the labeling of ADP-saturated MF_1 by NBD-Cl so that $k_2(MF_1 \cdot ADP) = k_1(MF_1)$, whereas in the labeling of ADP-saturated BF_1 by NBD-Cl, $k_2(BF_1 \cdot ADP) > k_1(BF_1)$ because of the larger ADP-induced shift in the reactivity vs. pH curve of BF_1 (Figure 3) as compared to MF_1 (data not shown). The observed rate constants for labeling of ATP-saturated enzymes by NBD-Cl, $k_2(MF_1 \cdot ATP) < k_1(MF_1)$ and $k_2(BF_1 \cdot ATP) \approx k_1(BF_1)$, may be due to the greater stereochemical interference of the bound ATP for this reaction. Stereochemical interference is probably the dominant factor in the labeling of P_i-saturated MF_1 and BF_1 by NBD-Cl, since the bound P_i retards this labeling reaction for both enzymes, i.e., $k_2(F_1 \cdot ADP) > k_2(F_1 \cdot ATP) > k_2(F_1 \cdot P_i)$. For the labeling of BF_1 by FDNB, $k_1 > k_2(BF_1 \cdot ADP) > k_2(BF_1 \cdot ATP)$. The rate of this labeling reaction is not enhanced by ADP, possibly because of greater stereochemical interference with FDNB. Thus, the present data on BF_1 are consistent with the previous suggestion based on studies of MF_1 that the reactive phenol group is located near the bound P_i or the γ-phosphate group of bound ATP at the catalytic site (Ting & Wang, 1980b).

Table V shows that for the labeling of BF_1 by TNBS, $k_1(BF_1) > k_2(BF_1 \cdot P_i)$. These values are also consistent with our previous suggestion that the essential amino group is located near the bound P_i or the γ-phosphate group of bound ATP at the catalytic site (Ting & Wang, 1981).

For the labeling of BF_1 by PG, the observed rate constants $k_1 \approx k_2(BF_1 \cdot Mg) > k_2(BF_1 \cdot ADP) \approx k_2(BF_1 \cdot ATP)$ are likewise consistent with the previous suggestion that the essential guanidinium group is probably located near the α- and β-phosphate group of bound ATP.

Lastly, for the labeling of BF_1 by DCCD, Table V shows that $k_1 \approx k_2(BF_1 \cdot ADP) > k_2(BF_1 \cdot ATP) > k_2(BF_1 \cdot Mg)$. These values are again consistent with the previous suggestion that the essential carboxyl group is probably located at the catalytic site but farther away from the bound ADP and is complexed with a Mg^{2+} ion which it may share with the γ-phosphate group of bound ATP.

There is also evidence for another essential Tyr residue at the catalytic site of MF_1-ATPase. It can be labeled by FSBA and may play an important role in the binding of ATP to the catalytic site (Esch & Allison, 1978, 1979; Pietro, et al., 1979;

Schäfer et al., 1978; Ting & Wang, 1981).

The fact that BF_1 from *E. coli* and MF_1 from bovine heart have the identical set of essential amino acid residues suggests the participation of their functional groups in the ATPase reaction. A possible catalytic mechanism with the participation of these identified functional groups has been proposed, which involves proton transfer from the Lys and Tyr functional groups to the γ-phosphate group of bound ATP, followed by proton transfer from the bound water to the phenolate ion and synchronized nucleophilic attack by the resulting hydroxide ion at the γ-phosphorus atom of ATP (Ting & Wang, 1980b).

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