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Functional Groups at the Catalytic Site of F_1 Adenosine Triphosphatase†

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ABSTRACT: The protection of F_1 ATPase by inorganic phosphate, ADP, ATP, and magnesium ion against inactivation by 1-fluoro-2,4-dinitrobenzene, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, and 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline, respectively, has been investigated. Dissociation equilibrium constants and rate constants for the labeling reactions have been deduced from a quantitative treatment of the kinetic data. Comparison of these dissociation constants with each other and with the corresponding literature values indicates that the essential Tyr, Arg, Lys, and Glu or Asp residues are indeed located at the catalytic site of the enzyme. Examination of the rate constants for the labeling reactions in the presence of excess inorganic phosphate, ADP, ATP, or magnesium ion, respectively, suggests that the essential phenol and amino groups are located nearer to the bound inorganic phosphate or the γ-phosphate group than to the α- or β-phosphate group of the bound ATP, that the essential guanidinium group is 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 that the essential carboxylate group is located slightly farther away but complexed with a magnesium ion which it shares with the bound inorganic phosphate. A mechanism consistent with these topographical relationships is proposed for the catalytic hydrolysis and synthesis of ATP.

Recent studies on the mitochondrial coupling factor F_1 (Senior, 1975; Wagenvoord et al., 1977) showed a subunit stoichiometry of $\alpha_2\beta_2\gamma_2\delta_x\epsilon_2$, where x is either 1 or 2, with a twofold axis of symmetry (Amzel & Pederson, 1978) and four types of substrate binding sites (Slater et al., 1979). Two of these sites, classified as type III in β subunits, have weak affinity for ATP and ADP and are believed to be catalytic sites responsible for ATPase activity. Kasahara & Penefsky (1978) showed that bovine heart F_1 has one high-affinity phosphate binding site which is probably located at the position of the γ-phosphate group of bound ATP in one of the two catalytic sites.

Several functional groups of F_1 were identified by inactivation studies. Inactivation of F_1 by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)[1] was shown to be due to the labeling of a specific tyrosine residue (Ferguson et al., 1975a,b). A more specific labeling reagent for the tyrosine residue is [*p*-(fluorosulfonyl)benzoyl]-5′-adenosine (Esch & Allison, 1978). However, simultaneous assays of ATPase activity and NBD labeling as a function of time showed that the ATPase inactivation was 90% complete when only 1 mol of NBD label had been incorporated into the enzyme (Steinmeier & Wang, 1979). This observation shows that under proper experimental conditions the labeling of F_1 ATPase by NBD-Cl can be quite specific. Inactivation of F_1 ATPase by phenylglyoxal was shown to be due to chemical modification of a number of arginine residues (Marcus et al., 1976; Frigeri et al., 1977, 1978; Kohlbrenner & Cross, 1978). F_1 ATPase was also irreversibly inhibited by pyridoxal phosphate under illumination (Godinot et al., 1979). In the absence of illumination, the inactivation of the ATPase by pyridoxal phosphate was incomplete (L. P. Ting and J. H. Wang, unpublished experiments), presumably due to the reversibility of the condensation reaction between the aldehyde and the essential Lys amino group of the enzyme. Inhibition of F_1 ATPase by 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) was found to be due to chemical modification of carboxyl groups which include at least one essential carboxyl group (Pougeois et al., 1978).

Inactivation of an enzyme by labeling a protein functional group does not necessarily imply the presence of the labeled group at the catalytic site, because the labeling reaction could have triggered a long-range protein conformation change with concomitant inactivation of the enzyme. However, recent investigations of the effect of binding of ATP, ADP, and P_i on the rate of labeling of F_1 ATPase by NBD-Cl and phenylglyoxal, respectively, suggest that the essential phenol and guanidinium groups are probably indeed at the catalytic site (Ting & Wang, 1980). In the present work, the protection of F_1 ATPase by P_i, Mg^{2+}, ATP, or ADP against inactivation by 1-fluoro-2,4-dinitrobenzene (FDNB) and EEDQ as well as NBD-Cl has been investigated. The kinetically deduced dissociation equilibrium constants and labeling rate constants

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; EEDQ, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; FDNB, 1-fluoro-2,4-dinitrobenzene; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (also named 4-chloro-7-nitrobenzofurazan); PEP, phosphoenolpyruvate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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have been examined with the aim to gain some insight regarding the topographical relationships of the functional groups with each other as well as with the bound substrate at the catalytic site.

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, and FDNB were from Sigma Chemical Co. [^{14}C]FDNB (21 mCi/mmol) was purchased from Amersham Corp. Sephadex G-50 fine was the product of Pharmacia Fine Chemicals, Inc. All other chemicals used were of reagent grade.

F_1 ATPase. Mitochondria were prepared from fresh bovine heart as described by Low & Vallin (1963). F_1 ATPase was prepared by the method of Knowles & Penefsky (1972). Different batches of F_1 had specific activities in the range 70–110 units/mg. Electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate and mercaptoethanol (Weber & Osborn, 1969) yielded five bands of approximate molecular weights 56 000, 51 000, 33 000, 14 000, and 8000, respectively. The F_1 preparation was stored at 5 °C as precipitate in a buffer containing 0.25 M sucrose, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 4 mM ATP, and 2 M ammonium sulfate. Shortly prior to use, an aliquot of the F_1 suspension was centrifuged at 7800*g* for 10 min, and the pellet was redissolved in either Hepes–EDTA–glycerol buffer or Hepes–glycerol buffer to a protein concentration of approximately 20 mg/mL. This protein solution was then desalted in a Sephadex G-50 fine column, 1 × 30 cm, which had been preequilibrated with the same buffer. The desalted F_1 had an A_{280}/A_{260} ratio of 1.55. The specific activity of the glycerol-stabilized F_1 ATPase solution decreased to 75% in the first few hours and then remained fairly constant during the next 24 h.

ATPase Assay. ATPase assay was performed in 3.0 mL of a solution containing 50 mM Tris–acetate, 3 mM $MgCl_2$, 2 mM ATP, 2 mM PEP, 0.2 mM NADH, 100 μg of pyruvate kinase, and 40 μg of L-lactic dehydrogenase at pH 7.5 and 30 °C. The absorbance change at 340 nm was followed in a Gilford Model 250 spectrophotometer. Under the experimental conditions, the rate of ATP hydrolysis is rate limiting, because the observed rate was proportional to the concentration of F_1 ATPase.

Protein Concentration. The concentration was determined by Bio-Rad protein assay, which employs a dye-binding method with Coomassie Blue G-250 as described by Bradford (1976). Bovine γ-globulin from Bio-Rad was used as a standard. The protein concentration so obtained was then corrected for a systematic error by calibration against the biuret method with bovine serum albumin as the primary standard.

Rate of Inactivation of F_1 ATPase by FDNB, NBD-Cl, and EEDQ. In each measurement of the rate of inactivation of F_1 ATPase by FDNB or NBD-Cl, 2 μL of an ethanolic stock solution of FDNB or NBD-Cl was added to 100 μL of a solution of F_1 in Hepes buffer (50 mM, pH 8.0) containing 25 mM NaCl, 2 mM EDTA, and 25% glycerol. The mixture was subsequently incubated at 24.5 °C in the dark, and aliquots were taken out at intervals for ATPase assay. In the control experiment, 2 μL of ethanol was added to the F_1 solution.

In the experiments for studying the effect of P_i, ATP, ADP, or Mg^{2+} on the rate of inactivation of F_1 by FDNB or NBD-Cl, the buffered F_1 solution was preincubated with the particular ligand or Mg^{2+} for 1 h at room temperature. Subsequently, F_1 was incubated with FDNB or NBD-Cl at 24.5 °C in the dark and assayed at intervals as before.

For the inactivation of F_1 ATPase by EEDQ, 2 μL of a methanolic solution of EEDQ was added to 100 μL of F_1 solution in 50 mM Hepes buffer (50 mM, pH 7.0) containing 25 mM NaCl and 25% glycerol with or without 2 mM EDTA. All other conditions were the same as those for the FDNB-labeling experiments.

A solution of F_1 was divided into two equal portions to study the effect of NBD label on the rate of labeling of the essential amino or carboxylate group of F_1 by FDNB or EEDQ. To one portion was added a calculated volume of 10 mM NBD-Cl solution in ethanol sufficient to reach a final concentration of 100 μM in NBD-Cl. To the second portion, used as control, was added an equivalent volume of ethanol. After incubation at 24.5 °C for 1.5 h in the dark, the portion with NBD-Cl no longer had ATPase activity. The two samples were then freed from small solute molecules by elution–centrifugation (Penefsky, 1977) to free the NBD–F_1 sample from excess NBD-Cl. Both the F_1 control and the NBD–F_1 samples were subsequently mixed with the second labeling reagent and incubated at 24.5 °C. Aliquots of both mixtures were then taken out at invervals and injected into assay mixtures containing 2 mM DTT for ATPase assay. The DTT was used to remove the NBD label from the essential Tyr residue and to restore the lost ATPase activity due to NBD labeling.

Correlation of [^{14}C]DNP Label on F_1 and NBD–F_1 with Their ATPase Activities. Samples of F_1 and NBD–F_1 were incubated with [^{14}C]FDNB. Aliquots were taken out at intervals from both incubation mixtures and freed from excess [^{14}C]FDNB by elution–centrifugation. The separated enzyme samples were used for ATPase assay, measurement of radioactivity, and determination of protein concentration.

Results

The rates of inactivation of F_1 ATPase by an excess of 1-fluoro-2,4-dinitrobenzene (FDNB) have been measured. At any given concentration of the labeling reagent, the decay of ATPase activity followed first-order kinetics. The rate of inactivation is also first order with respect to [FDNB] since a plot of log $t_{1/2}$ against log [FDNB] gives a straight line of slope −0.84. Consequently, the rate of inactivation may be written as

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

where R represents the concentration of the labeling reagent.

The experimental data in Figure 1 show that P_i can protect F_1 against inactivation by FDNB. At any given concentration of the excess reagent, the observed rate of inactivation may be considered as the sum of the labeling rates of free F_1 and of F_1 with P_i bound at the catalytic site, respectively, by FDNB in solution, i.e.

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

and hence the familiar expression

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

where K_P represents the dissociation constant for P_i bound to

Table I: Effect of Phosphate, Adenine Nucleotides, and Mg^{2+} on the Inactivation of F_1 by FDNB[a]

expt	$t_{1/2}$ (min)
control	6.5
+4.55 mM ATP	12.7
+4.55 mM ADP	8.0
+4.55 mM P_i	12.0
+4.55 mM $MgCl_2$	6.4

[a] Incubation conditions: 1.2 μM F_1 in 50 mM Hepes, 25 mM NaCl, 2 mM EDTA, and 25% glycerol, pH 8.0, was preincubated with additions at 24.5 °C for 1 h. Then the preincubated F_1 was inactivated by 0.98 mM FDNB. A fairly high concentration of ATP or ADP was used to ensure complete binding at the catalytic site. For the experimental K_m values, see Table IV.

this FDNB-sensitive site in F_1, and k_1 and k_2 represent the apparent rate constants for the inactivation reaction with $[P_i] \ll K_P$ and $[P_i] \gg K_P$, respectively. Integration of eq 3 gives

$$\ln [F_1]/[F_1]_0 = -k'Rt \quad (4)$$

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 [FDNB]. Such a linear plot is shown in the inset of Figure 1. The values so determined for the labeling of F_1 by FDNB are $k_1 = 0.11$ mM^{-1} min^{-1}, $k_2 = 0.048$ mM^{-1} min^{-1}, and $K_P = 1.1$ mM at 24.5 °C in a buffered solution at pH 8.0 containing 25 mM NaCl but no Mg^{2+}. This value of K_P is within experimental error, in agreement with the previous values $K_P = 1.3$ and 1.4 mM determined through the protection of F_1 ATPase by P_i against inactivation by NBD-Cl and phenylglyoxal, respectively, in the absence of Mg^{2+} (Ting & Wang, 1980). In order to determine the dissociation constant of P_i bound to F_1 in the presence of Mg^{2+}, the rates of inactivation of F_1 ATPase by NBD-Cl in a solution containing a constant concentration of Mg^{2+} (50 mM Hepes, 25 mM NaCl, 2 mM EDTA, 25% glycerol, 2.94 mM $MgCl_2$, 1.2 μM F_1, and 49 μM NBD-Cl at 24.5 °C) but different concentrations of P_i (0–1.76 mM) were measured. The data when treated similarly give a value of $K_{MgP} = 0.43$ mM. Kasahara & Penefsky (1977, 1978) showed that the binding of P_i by F_1 is independent of temperature and stimulated by Mg^{2+}. These

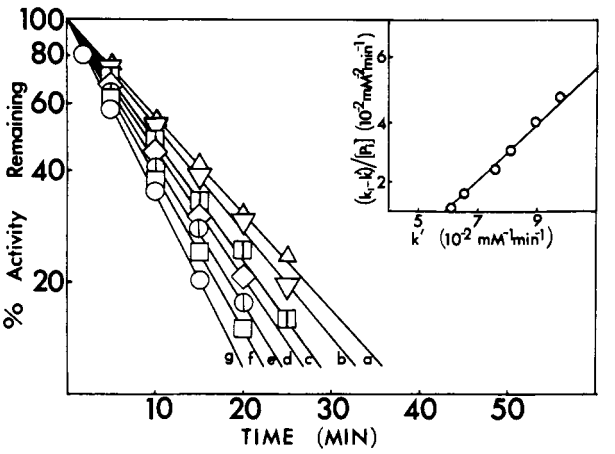

FIGURE 1: Effect of different concentrations of P_i on the inactivation of F_1 by FDNB. The reaction conditions were the same as in Table I. Concentrations of P_i are (a) 4.50, (b) 2.73, (c) 1.36, (d) 0.91, (e) 0.45, (f) 0.23, and (g) 0 mM. The inset shows the determination of the dissociation constant K_P of bound P_i at the active site in F_1. The slope of this linear plot gives $K_P = 1.1$ mM.

investigators obtained a value of $K_{MgP} = 0.29$ mM in a buffered solution at pH 8.1 containing 1 mM $MgSO_4$ but no NaCl.

Table I shows that P_i or ATP effectively protects and ADP slightly protects whereas Mg^{2+} by itself does not protect F_1 ATPase against inactivation by FDNB. Kinetic data on the protection of F_1 ATPase by ATP or ADP alone against inactivation by FDNB could presumably be treated in a way similar to eq 1–6 to determine K_{ATP}, K_{ADP}, k_1, $k_2(F_1{\cdot}ATP)$, and $k_2(F_1{\cdot}ADP)$. But the approximate values $k_2 \approx 0.05$ mM^{-1} min^{-1} for $F_1{\cdot}ATP$ and $k_2 \approx 0.09$ mM^{-1} min^{-1} for $F_1{\cdot}ADP$ were obtained directly from the saturation $t_{1/2}$ values in Table I.

The 2,4-dinitrophenyl (DNP) labels are probably all covalently attached to ε-amino groups of Lys, since the terminal amino groups of F_1 ATPase seem blocked (Senior, 1973). In order to distinguish specific from nonspecific labeling of the enzyme by FDNB, the inactivation of F_1 ATPase by [^{14}C]FDNB was followed by both the decrease of enzyme activity and the increase of radioactivity of the protein. Parallel inactivation experiments of NBD–F_1 by [^{14}C]FDNB were also conducted in which the enzyme activity of each sample was assayed after removal of the NBD label with DTT. The results summarized in Table II show that the rate of inactivation and the rate of radioactive labeling of NBD–F_1 are both slower than those for F_1, indicating that the specific NBD label on the essential Tyr phenol group hinders the labeling of the Lys amino group. If it can be assumed that the NBD label has a negligible effect on the rate of nonspecific labeling, then the difference in fractions of enzyme activity lost between F_1 and

Table II: Labeling and Inactivation of F_1 and NBD–F_1 by FDNB[a]

incubn time (min)	F_1		NBD–F_1		diff in fractions of act. lost between F_1 and NBD–F_1	diff in radioact labeling between F_1 and NBD–F_1 (cpm/pmol)	sp labeling for complete inhibn (cpm/pmol of enzyme)
	fraction of act. lost	radioact (cpm/pmol)	fraction of act. lost	radioact (cpm/pmol)			
0	0	0	0	0			
10	0.35	36.2	0.14	27.7	0.21	8.5	40.5
20	0.59	60.5	0.23	46.0	0.36	14.5	40.3
30	0.77	77.1	0.35	61.0	0.42	16.1	38.3
45	0.87	98.7	0.45	83.1	0.42	15.6	37.1
60	0.91	113.1	0.54	99.5	0.37	13.6	36.8

[a] Incubation conditions: 5.1 μM F_1 and 5.1 μM NBD–F_1 in 50 mM Hepes, 25 mM NaCl, 2 mM EDTA, and 25% glycerol, pH 8.0, were respectively labeled and inactivated at 24.5 °C by 0.3 mM radioactive FDNB with a specific radioactivity of 21 μCi/μmol, corresponding to 41 cpm/pmol FDNB.

Table III: Effect of Phosphate, Adenine Nucleotides, and Mg^{2+} on the Inactivation of F_1 by EEDQ[a]

expt	EDTA (mM)	pH	$t_{1/2}$ (min)
F_1	2	7.0	5.0
+4.55 mM ATP	2	7.0	4.7
+4.55 mM ADP	2	7.0	5.0
+4.55 mM P_i	2	7.0	5.1
+1.56 mM Mg^{2+}	2	7.0	8.7
+1.56 mM Mg^{2+} + 1.56 mM P_i	2	7.0	8.7
+1.56 mM Mg^{2+} + 3.12 mM P_i	2	7.0	8.7
+4.55 mM Mg^{2+}	2	7.0	11.3
+4.55 mM Mg^{2+} + 4.55 mM P_i	2	7.0	11.3
F_1	0	6.9	7.5
F_1	2	6.9	3.8
+0.30 mM Mg^{2+}	0	6.9	7.6
+1.14 mM Mg^{2+}	0	6.9	7.5
+4.55 mM Mg^{2+}	0	6.9	7.6
+4.55 mM P_i	0	6.9	7.7
+4.55 mM ADP	0	6.9	4.4
+4.55 mM ATP	0	6.9	3.6

[a] Incubation conditions: 1.2 μM F_1, NBD–F_1, or preincubated F_1 in 50 mM Hepes, 25 mM NaCl, 25% glycerol, and EDTA, pH as indicated, was inactivated at 24.5 °C by 1.96 mM EEDQ.

NBD–F_1 must be due to the difference in the extent of specific labeling. Likewise, the difference in radioactive labeling between F_1 and NBD–F_1 must also be due to the difference in the extent of specific labeling. Values of the extent of specific labeling required for complete inhibition of F_1 ATPase by FDNB, shown in the last column of Table II, were computed by dividing the values in the seventh column by the corresponding values in the sixth column of Table II. The approximate constancy of the values in the last column substantiates our assumption. Since the specific radioactivity of the [^{14}C]FDNB is 41 cpm/pmol, we conclude that complete inactivation of F_1 ATPase by FDNB requires the labeling of only one Lys amino group per F_1 molecule.

The observed inhibition of F_1 ATPase by 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) suggests that among the labeled carboxyl groups there is at least one essential carboxyl group in the enzyme (Pougeois et al., 1978). Kinetic data show that the rate of inactivation of F_1 ATPase by different concentrations of EEDQ is also given by eq 1 and hence can be analyzed in the same way. The data summarized in Table III show that in the presence of 2 mM EDTA the inactivation of F_1 by EEDQ is inhibited by Mg^{2+}, but not significantly affected by P_i, ADP, or ATP. However, the data also show that in the absence of EDTA the reaction is accelerated by ATP or ADP but not affected by Mg^{2+} or P_i.

This seemingly anomalous behavior is probably due to inhibition of the EEDQ reaction in the absence of EDTA by endogenous Mg^{2+} bound to the catalytic site in the F_1 preparation. Apparently, 2 mM EDTA is sufficient to remove all the endogenous Mg^{2+} bound to the catalytic site of F_1 to give the uninhibited rate of EEDQ inactivation ($t_{1/2}$ = 5 min for R = 1.96 mM). The observed decrease in the rate of inactivation after the addition of exogenous Mg^{2+} was due presumably to the complexation of Mg^{2+} with the essential carboxylate group of F_1. In the absence of EDTA, this essential carboxylate group is presumably already fully coordinated to endogenous Mg^{2+}. Consequently, further addition of exogenous Mg^{2+} to the incubation mixture produced no effect, but removal of endogenous Mg^{2+} from F_1 by competing ligands such as ATP or ADP increases the rate of EEDQ inactivation as shown in Table III.

Kinetic data on the protection of F_1 ATPase in 2 mM EDTA solution by Mg^{2+} against inactivation by EEDQ are

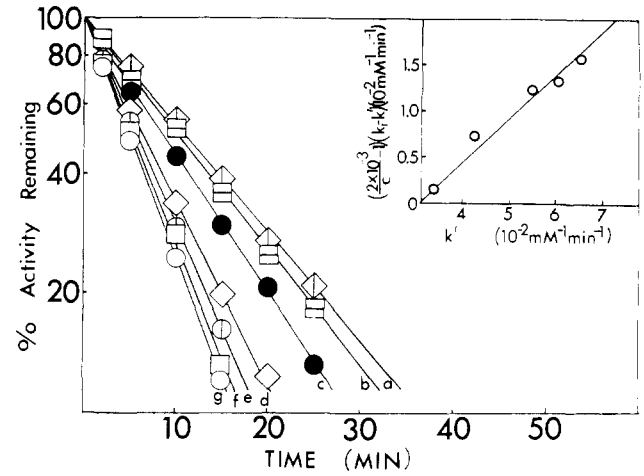

FIGURE 2: Effect of different concentrations of Mg^{2+} on the inactivation of F_1 by EEDQ. The reaction conditions were 50 mM Hepes, 25 mM NaCl, 2 mM EDTA, 25% glycerol, pH 7.0, 1.2 μM F_1, and 1.96 mM EEDQ at 24.5 °C. The concentrations of Mg^{2+} are (a) 4.55, (b) 1.92, (c) 1.56, (d) 1.14, (e) 0.93, (f) 0.57, and (g) 0 mM. The inset shows a plot according to eq 11 for determining the values of K_{Mg} and k_2.

summarized in Figure 2. In order to treat the data quantitatively, let us consider the equilibria in solution with total magnesium concentration below 2 × 10^{-3} M:

$$EDTA\text{–}Mg^{2+} \rightleftarrows EDTA + Mg^{2+}$$

$$K' = (2 \times 10^{-3} - c)x/c \quad (7)$$

$$F_1\text{–}Mg^{2+} \rightleftarrows F_1 + Mg^{2+}$$

$$K_{Mg} = (a - y)x/y \quad (8)$$

where $x = [Mg^{2+}]_{free}$, $y = [F_1\text{–}Mg^{2+}]$, and $a = [F_1\text{–}Mg^{2+}] + [F_1]$, and since $[F_1\text{–}Mg^{2+}] \ll [EDTA\text{–}Mg^{2+}] \gg [Mg^{2+}]$, we have $c = [EDTA\text{–}Mg^{2+}] + [F_1\text{–}Mg^{2+}] + [Mg^{2+}] \approx [EDTA\text{–}Mg^{2+}]$. Elimination of x from eq 7 and 8 gives

$$\frac{a - y}{a} = \frac{(2 \times 10^{-3} - c)(K_{Mg}/K')}{(2 \times 10^{-3} - c)(K_{Mg}/K') + c} \quad (9)$$

With the same notation as in eq 1–6, we obtain

$$-\frac{d \ln [F_1]}{dt} = \left\{k_1\left(\frac{a - y}{a}\right) + k_2\left(\frac{y}{a}\right)\right\}R = k'R \quad (10)$$

Combination of eq 9 and 10 gives

$$[(2 \times 10^{-3}/c) - 1](k_1 - k') = (K'/K_{Mg})(k' - k_2) \quad (11)$$

Again, since k_1 can be determined in a control experiment without added Mg^{2+} and k' is the observed apparent rate constant for inactivation of F_1 ATPase by EEDQ, the values of k_2 and K'/K_{Mg} can be determined from the linear plot of the left-hand side of eq 11 vs. k'. The plot in the inset of Figure 2 gives k_2 = 0.032 mM^{-1} min^{-1} and K'/K_{Mg} = 0.45. Since the apparent dissociation constant of EDTA–Mg^{2+} at pH 7.0 is K' = 3.98 μM (Dawson et al., 1969), we obtain K_{Mg} = 8.9 μM.

In contrast to the reaction with FDNB, it was found that F_1 and NBD–F_1 were labeled by EEDQ at the same rate. For example, in 50 mM Hepes buffer at pH 7.5, 24.5 °C, containing 25 mM NaCl, 2 mM EDTA, and 25% glycerol, the half-time of reaction with 1.96 mM EEDQ was found to be

Table IV: Summary of Kinetic and Dissociation Constants for F_1 ATPase[a]

constant	labeling reagent			
	NBD-Cl	phenylglyoxal	FDNB	EEDQ
K_P (mM)	1.3	1.4	1.1	
K_{ADP} (μM)	210	160		
K_{Mg} (μM)				8.9
K_{MgP} (mM)	0.43			
k_1 (mM^{-1} min^{-1})	3.5	0.022	0.11	0.072
$k_2(F_1 \cdot P_i)$ (mM^{-1} min^{-1})	0.27	0.011	0.048	0.07
$k_2(F_1 \cdot MgP_i)$ (mM^{-1} min^{-1})	0			0.032
$k_2(F_1 \cdot ADP)$ (mM^{-1} min^{-1})	3.5	0.005	~0.088	0.07
$k_2(F_1 \cdot ATP)$ (mM^{-1} min^{-1})	~1.8	0.005	~0.06	0.07
$k_2(F_1 \cdot Mg^{2+})$ (mM^{-1} min^{-1})	3.5	0.022	0.11	0.032

[a] All measurements were conducted at 24.5 °C in 50 mM Hepes buffer containing 25 mM NaCl, 2 mM EDTA, and 25% glycerol.

13.3 min for both F_1 and NBD–F_1.

Discussion

The results obtained in this work on the effect of P_i, ADP, ATP, and Mg^{2+} on the inactivation of F_1 ATPase by FDNB, NBD-Cl, and EEDQ are summarized in Table IV together with similar data obtained previously with phenylglyoxal as well as NBD-Cl (Ting & Wang, 1980). The observation that F_1 ATPase is inactivated when it is covalently labeled with NBD-Cl, phenylglyoxal, FDNB, and EEDQ, respectively, does not necessarily indicate the presence of phenol, guanidinium, amino, and carboxylate groups at the catalytic site, because each of these labeling reactions could have triggered a long-range protein conformation change which inactivates the enzyme. However, it seems very improbable that three of the structurally unrelated labeling reactions could have triggered the same extent of long-range protein conformation change such that the values of K_P determined from labeling measurements with NBD-Cl, phenylglyoxal, and FDNB, respectively, as listed in Table IV turned out to be equal within experimental uncertainties. Table IV also shows that the value K_{MgP} = 0.43 mM determined from the rate of labeling with NBD-Cl is compatible with the value K_{MgP} = 0.29 mM determined by Kasahara & Penefsky (1978), and the values K_{ADP} = 210 and 160 μM determined from labeling with NBD-Cl and phenylglyoxal, respectively, agree with the value K_{ADP} = 140 μM determined by ultrafiltration for the catalytic site (Wielders et al., 1980). It seems much simpler to explain the observed protection of F_1 ATPase by P_i, ADP, and ATP against inactivation by NBD-Cl, phenylglyoxal, and FDNB, respectively, by assuming that these labeled essential functional groups are indeed at the catalytic site of the enzyme.

According to Table IV, bound P_i decreases the rate constant for the specific labeling of F_1 ATPase by NBD-Cl by a factor of 13, but bound ADP has no effect. However, ATP does have an appreciable effect. The simplest explanation of these observations seems to be that the essential Tyr phenol group is located closer to the bound P_i or the γ-phosphate than to the α- or β-phosphate group of the bound ATP at the catalytic site. Similarly, the experimental values of k_2 for specific labeling of F_1 ATPase by FDNB suggest that the essential amino group at the catalytic site is also located closer to the bound P_i or the γ-phosphate than to the α- or β-phosphate group of the bound ATP.

On the other hand, the k_2 values for labeling the essential guanidinium group at the catalytic site suggest that this functional group is located closer to the α- or β-phosphate group than to the γ-phosphate group of the bound ATP or to the bound P_i.

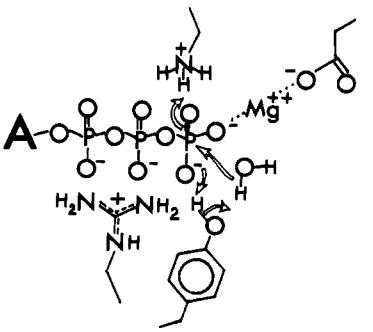

FIGURE 3: Functional groups at the catalytic site of F_1 ATPase and their topographical relationships to each other as well as to the bound substrate as suggested by chemical and kinetic data. The hollow arrows indicate a catalytic mechanism for the hydrolysis of ATP.

The essential carboxylate group of F_1 ATPase cannot be located as easily because its reaction with EEDQ in the presence of EDTA is unaffected by P_i, ADP, or ATP (Table III). It is probably not in direct van der Waals contact with the essential phenol group because F_1 and NBD–F_1 react with EEDQ at the same rate. But Mg^{2+} does protect F_1 ATPase against inactivation by EEDQ, presumably due to its complexation with the essential carboxylate group. Although Mg^{2+} by itself does not protect F_1 ATPase against inactivation by NBD-Cl, Table IV shows that the observed dissociation constant of the bound P_i in the presence of Mg^{2+} (K_{MgP}) is only one-third of its value in the absence of Mg^{2+} (K_P); i.e., Mg^{2+} can enhance the protection of the enzyme by low concentrations of P_i against inactivation by NBD-Cl. This observation suggests that the Mg^{2+} ion is probably ligated by both P_i and the essential carboxylate group so that it assists in binding P_i to the catalytic site.

The essential Tyr phenol and Lys amino groups are probably not far from each other because of the ease of migration of the NBD label from the phenolate to the amino group at pH >8 (Ferguson et al., 1975a,b). The essential phenol and guanidinium groups are probably also not far from each other because of the observed enhancement in the rate of labeling of the guanidinium group by the NBD label on the phenolate group (Ting & Wang, 1980).

The topographical relationships of the four identified functional groups with each other as well as with the bound substrate at the catalytic site of the F_1 ATPase as suggested by chemical and kinetic data are summarized in Figure 3. A plausible catalytic mechanism for ATP hydrolysis involves direct proton transfer from the Lys and Tyr functional groups to the γ-phosphate group of the bound ATP as indicated by the hollow arrows. This is immediately 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 to form the trigonal-bipyramidal intermediate.

The catalytic mechanism for ATP synthesis driven by proton flux (Mitchell, 1961) is even more intriguing, for there seems to be no known example in organic chemistry in which the product of a reaction is determined by the relative direction of diffusion of the reactants. Mitchell (1977) proposed the "phosphoryllium" ion PO_2^+ as a reaction intermediate in ATP synthesis catalyzed by the $F_0 \cdot F_1$ complex. Such an intermediate seems difficult to reconcile with our present knowledge in chemistry. However, a feasible mechanism can be contemplated by assuming the ADP + P_i pair to be bound to the catalytic site in such a way that protons arriving through the F_0 channel can rapidly protonate the bound P_i without protonating the bound nucleophile ADP which could effectively

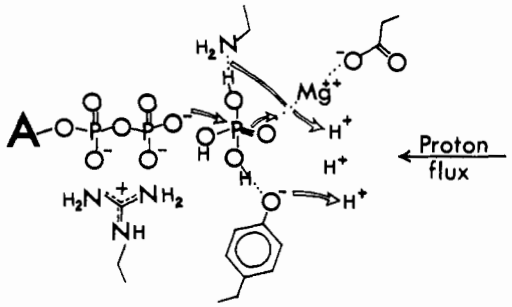

FIGURE 4: A catalytic mechanism for the phosphorylation of ADP driven by proton flux suggested by chemical and kinetic data. Protonation of the functional amino and phenolate groups could effectively prevent the trigonal-bipyramidal active intermediate from going back to the reactant state and facilitate the elimination of water.

attack the fully activated phosphate. Such a catalytic mechanism is illustrated in Figure 4 which may represent either the same catalytic site as that illustrated in Figure 3 but in the phosphorylation conformation according to the alternating-site model (Adolfsen & Moudrianakis, 1976; Kayalar et al., 1977) or the phosphorylation site with a similar set of functional groups according to the separate-sites model (Penefsky, 1974a,b; Pedersen, 1975). Initially, the HPO_4^{2-} ion may be bound to the essential carboxylate group through a shared Mg^{2+} ion as well as to the functional groups of the Tyr and Lys through hydrogen bonds and ion-pair interaction. The transient, fully activated P_i, shown in Figure 4 could be produced by rapid proton transfer from the phenol group and the protonated amino group along the indicated hydrogen bonds during the first activation step. It should be highly reactive and susceptible to the protein conformation-assisted nucleophilic attack by the unprotonated ADP to form the trigonal-bipyramidal intermediate, leading to ATP and H_2O. Normally, the yield of ATP would be negligible because of the more favorable reverse reaction. But protons arriving through the F_0 channel could rapidly protonate a number of protein functional groups including the transient phenolate and basic amino groups, thereby effectively preventing the trigonal-bipyramidal intermediate from going back to the reactant state and facilitating the elimination of water. In this way, the condensation of ADP with P_i could be driven to completion by the proton flux. Because of its high pK_a, the guanidinium group appears to be a good choice for helping to bind the triply negatively charged ADP without protonating this nucleophile.

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