Journal of Bioenergetics and Biomembranes, Vol. 12, Nos. 3/4, 1980

Effect of Phosphate and Adenine Nucleotides on the Rate of Labeling of Functional Groups at the Catalytic Site of F_1-ATPase[1]

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Received December 27, 1979

Abstract

The effect of inorganic phosphate, ADP, ATP, and their analogues on the rate of labeling of F_1-ATPase by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and phenylglyoxal have been investigated. Analysis of the kinetic data indicate that the labeled functional groups of the essential tyrosine and arginine residues respectively are both located at the catalytic site of F_1. The active phenolic group of tyrosine is located closer to the bound inorganic phosphate or the γ-phosphate group than the α- and β-phosphate groups of the bound ATP at the catalytic site, whereas the guanidinium group of arginine is located closer to the α- and β-phosphate groups of the bound ATP than to its γ-phosphate group or the bound inorganic phosphate. The kinetically deduced dissociation constants are 1.3 mM and 210 μM for the inorganic phosphate and ADP respectively bound to this catalytic site. Labeling the essential tyrosine residue by NBD-Cl has been found to facilitate subsequent labeling of the essential arginine residue by phenylglyoxal.

Introduction

The terminal enzyme of oxidative phosphorylation is generally considered to consist of two sectors, a sector designated as F_1 which is easily detachable from the membrane and another sector designated as F_0 which is an integral

[1]Abbreviations: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (this compound has been named 4-chloro-7-nitro-benzofurazan and abbreviated NBf-Cl elsewhere); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; PEP, phosphoenolpyruvate; ADPCP, β,γ-methylene-adenosine 5'-triphosphate; AMPCP, α,β-methylene-adenosine 5'-diphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, 2-amino-2(hydroxymethyl)-1,3-propanediol.

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part of the inner membrane. The properties of F_1 have recently been reviewed by Pedersen [1, 2], Nelson [3], Kozlov and Skulachev [4], Harris [5] and Hammes [6]. Studies by Senior [7] and Wagenvoord et al. [8] support a subunit stoichiometry of $\alpha_2\beta_2\gamma_2\delta_x\epsilon_2$, where x is presumably 1 or 2. Preliminary crystallographic studies by Amzel and Pedersen have revealed a two-fold symmetry [9].

Recently Slater et al. [10] showed that bovine heart mitochondrial F_1 has four types of nucleotide binding sites. Of these, Type I and Type II are nucleotide binding sites with strong affinity for ATP and ADP respectively. The two Type III sites in β-subunits have weak affinity for ATP and are believed to be catalytic sites responsible for ATPase activity. Lastly, there are two anion sites (Type IV) in the α-subunits which are believed to have regulatory functions. However, Kasahara and Penefsky [12, 13] have shown that bovine heart mitochondria F_1 has one high-affinity phosphate binding site which is probably located at the position of γ-phosphate of ATP in one of the catalytic sites. In the conformation of isolated F_1, ADP cannot be bound simultaneously with P_i and hence can prevent P_i from binding at this site. NBD-Cl and phenylglyoxal have been shown to inactivate the ATPase through chemical modification of specific tyrosine [14, 15] and arginine [16–19] residues respectively. On the other hand, Esch and Allison [20] succeeded in labeling an essential tyrosine residue of F_1 with *p*-fluorosulfonyl-[C^{14}]benzoyl-5′-adenosine, although they observed a different pH dependence of the labeling rate from the pH dependence of the labeling rate by NBD-Cl reported by Ferguson et al. [14]. It is of interest to know whether the tyrosine modified by NBD-Cl and the arginine modified by phenylglyoxal are at the same catalytic site and, if so, how are they topographically related to the bound ATP.

The results presented in this paper strongly suggest that both the NBD-Cl-modified tyrosine and the phenylglyoxal-modified essential arginine are at the catalytic site and that this tyrosine residue is close to the γ-phosphate group of the bound ATP, whereas the arginine residue is close to the α- and β-phosphate groups of the bound ATP.

Materials and Methods

Materials

Pyruvate kinase (Type II), L-lactic dehydrogenase (Type III), phosphoenolpyruvate, ATP (equine muscle), ADP (Grade 1), ADPCP, AMPCP, NADH (Grade III), NBD-Cl, and phenylglyoxal were purchased from Sigma Chemical Company. [^{14}C]NBD-Cl (109 Ci/mole) and [^{14}C]phenylglyoxal (25 Ci/mole) were purchased from Research Products International

Corporation. Sephadex-G-50 was a product of Pharmacia Fine Chemicals, Inc. All other chemicals used were of the reagent grade.

Preparation of F_1. Mitochondria were prepared from fresh beef heart essentially as described by Löw and Vallin [21]. F_1 was prepared by the method of Knowles and Penefsky [22]. The specific activity of ATPase of F_1 was in the range of 80 to 110 units/mg. On polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and mercaptoethanol [23], the purified F_1 yielded five bands of approximate molecular weights 56,000, 51,000, 33,000, 14,000, and 8,000 respectively. The F_1 was stored at +5°C as a precipitate in buffer containing 0.25 M sucrose, 50 mM Tris-Cl, 2 mM EDTA, 4 mM ATP, pH 8.0, and 2 M ammonium sulfate. Shortly prior to use, an aliquot of the F_1 suspension was centrifuged at 10,000 rpm for 10 min and the pellet was redissolved in either Hepes–EDTA–glycerol buffer (50 mM Hepes, 2 mM EDTA, 25% glycerol, pH 8.0) or triethanolamine–EDTA–glycerol buffer (50 mM triethanolamine, 2 mM EDTA, 25% glycerol, pH 8.0) to a protein concentration of approximately 20 mg/ml. The protein solution was later applied to a Sephadex-G-50-fine column, 1 × 30 cm, equilibrated with the same buffer for desalting. The desalted F_1 has an A_{280nm}/A_{260nm} ratio of 1.55.

ATPase Assay

ATPase assay was carried out at 30°C in a total volume of 3.0 ml 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. Absorbance change at 340 nm was followed in a Gilford Model 250 Spectrophotometer.

Protein Concentration

The protein concentration was determined by Bio-Rad protein assay which employs a dye-binding method using Coomassie Blue G-250 as described by Bradford [24]. Bovine gamma globulin was used as a reference standard. The protein concentration so obtained was then corrected for systematic error by calibrating against the biuret method with bovine serum albumin as the primary standard.

Rate of Inactivation of F_1 by NBD-Cl and Phenylglyoxal

In the kinetic studies of the inactivation of F_1 by NBD-Cl, 2 μl of an ethanolic solution of NBD-Cl was added to 100 μl of a buffered solution of F_1 (50 mM triethanolamine hydrochloride or Hepes, 2 mM EDTA, 25 mM NaCl, and 25% glycerol, pH 8.0). The mixture was subsequently incubated at 23°C in the dark. 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

studies of the protection of F_1 by P_i, ADP, ATP, etc. against inactivation by NBD-Cl, F_1 in the buffered solution was preincubated with inorganic phosphate and/or nucleotides for 2 h at room temperature. Then the F_1 was incubated with NBD-Cl at 23°C in the dark and assayed at intervals as before.

For the inactivation of F_1 by phenylglyoxal, the experimental conditions were essentially the same except that the reaction mixture was incubated at 30°C and not in the dark.

In the study of the effect of NBD-labeling on the inactivation of F_1 by phenylglyoxal, a solution of F_1 was divided into two portions. To one portion was added a calculated volume of a 10 mM NBD-Cl solution in ethanol sufficient to reach a final concentration of 100 μM NBD-Cl. To the second portion, used as the control, was added an equivalent volume of ethanol. After a 2-h incubation period at 23°C in the dark, the portion with NBD-Cl no longer had ATPase activity. The two samples were subsequently freed from the excess of NBD-Cl by elution–centrifugation [25]. Each sample was applied to the top of a small column of Sephadex-G-50 (fine) in a 1-ml plastic tuberculin syringe placed in a centrifuge tube. Each purified sample was then mixed with phenylglyoxal and incubated at 30°C. Aliquots were taken out and injected into assay mixtures for ATPase activity. The assay mixture contained 2 mM DTT for removing the NBD-label from the tyrosine residue and restoring the ATPase activity.

Correlation of [^{14}C]NBD-label on F_1 with ATPase Activity

Aliquots were taken out of an incubation mixture of F_1 and [^{14}C]NBD-Cl for isolation of [^{14}C]NBD-F_1 and ATPase assay. The [^{14}C]NBD-F_1 was freed from unreacted [^{14}C]-NBD-Cl by elution–centrifugation. The eluate obtained after centrifugation was used for the determination of protein concentration, assay of ATPase activity, and radioactivity. The observed cpm values were corrected for background radioactivity determined by omission of the enzyme in the reaction mixture.

Results

Rate of Inactivation of F_1 by NBD-Cl

The rates of inactivation of the ATPase activity of F_1 at different NBD-Cl concentrations are shown in Fig. 1. The experimental linear plot of log $T_{1/2}$ against log [NBD-Cl] in the insert gives a slope of 1.06. In agreement with previous work [14, 26], it was observed that 1 mM DTT removed the NBD-label from NBD-F_1 and restored full ATPase activity within 20 sec after the addition of the thiol.

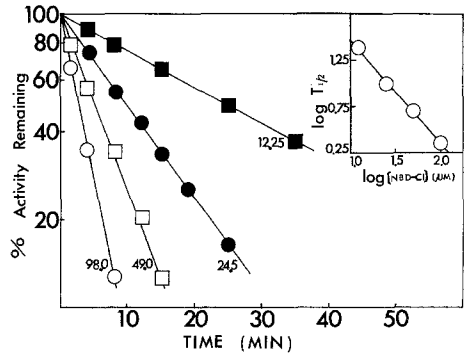

Fig. 1. Inactivation of F_1-ATPase activity by NBD-Cl. The reaction conditions were: 50 mM triethanolamine hydrochloride, 2 mM EDTA, 25% glycerol, pH 8.0, 23°C and 1.2 μM F_1. Concentrations of NBD-Cl in μM are indicated in the figure. The insert gives the order of the reaction with respect to NBD-Cl.

Protection of F_1 against Inactivation by NBD-Cl

The effect of different concentrations of P_i on the inactivation of F_1 by a large excess of NBD-Cl is shown in Fig. 2. The observed continual decrease in the inactivation rate as the concentration of P_i is raised suggests that P_i can protect F_1 against inactivation by NBD-Cl.

Since the rate of inactivation of F_1 is proportional to the concentration of NBD-Cl (Fig. 1), each of the inactivation experiments may be treated as the sum of reactions of NBD-Cl in solution with free F_1 and F_1 with P_i bound at the catalytic site ($F_1 \cdot P_i$) respectively. In each experiment with a large excess

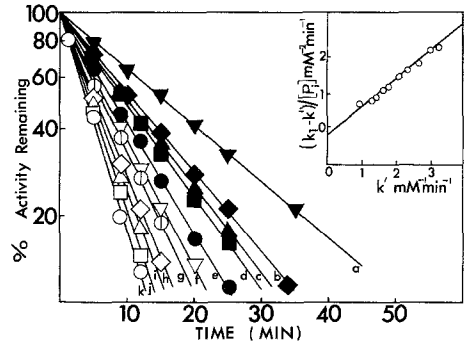

Fig. 2. Effect of different concentrations of P_i on NBD-Cl inactivation. Reaction conditions: 50 mM Hepes, 25 mM NaCl, 2 mM EDTA, 25% glycerol, pH 8.0, 23°C, 1.2 μM F_1, and 49 μM NBD-Cl. Concentrations of P_i are (a) 3.81 mM, (b) 2.86 mM, (c) 2.38 mM, (d) 1.91 mM, (e) 1.43 mM, (f) 0.95 mM, (g) 0.71 mM, (h) 0.48 mM, (i) 0.24 mM, (j) 0.12 mM, and (k) 0 mM. The insert shows the determination of the dissociation constant (K_P) of P_i bound at this active site in F_1. The details are given in the text. The experimentally determined values are: $K_P = 1.3$ mM, $k_1 = 3.5$ mM^{-1} min^{-1}, and $k_2 = 0.27$ mM^{-1} min^{-1}.

of NBD-Cl, the concentration of active F_1 should decrease with time as given by

$$-d[F_1]/dt = (k_1[F_1]_{free} + k_2[F_1 \cdot P_i])\,[\text{NBD-Cl}]$$

and hence

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

where K_P represents the dissociation constant for P_i bound to this NBD-Cl-sensitive site in F_1, and k_1 and k_2 the apparent first-order rate constants for the inactivation reaction with $[P_i] \ll K_p$ and $[P_i] \gg K_p$ respectively.

Integration of Eq. (1) gives

$$\ln ([F_1]/[F_1]_0) = k't\,[\text{NBD-Cl}]$$

where the apparent rate constant k' is given by

$$k' = k_1K_P/(K_P + [P_i]) + k_2[P_i]/(K_P + [P_i])$$

which, upon rearrangement, becomes

$$(k_1 - k')/[P_i] = (k' - k_2)/K_P \quad (2)$$

Since k_1 is the observed inactivation rate constant in the control experiment without P_i, K_p and k_2 can be readily determined from the slope and intercept of the linear plot of the observed values of $k_1 - k'$ versus k'. The plot in the insert of Fig. 2 gives $K_P = 1.3 \times 10^{-3}$ M, $k_1 = 3.5$ mM^{-1} min^{-1}, and $k_2 = 0.27$ mM^{-1} min^{-1} in a buffered solution at pH 8.0 and 23°C containing 25 mM NaCl but no Mg^{2+}. Kasahara and Penefsky [11–13] showed that the binding of P_i to F_1 is independent of temperature and stimulated by Mg^{2+}, and they obtained the value $K_P = 0.29 \times 10^{-3}$ M in a buffered solution at pH 8.1 containing 1 mM $MgSO_4$ but no NaCl.

As expected from their structural similarity, Fig. 3 shows that sulfate and arsenate can also protect F_1 against inactivation by NBD-Cl, though less effectively.

The effect of ADP and ATP in the absence or presence of P_i on inactivation by NBD-Cl is shown in Fig. 4. The rate of inactivation by NBD-Cl is decreased by 8.3 mM ATP, but not by 8.3 mM ADP. Also, 1 mM P_i + 8.3 mM ATP or 2.5 mM P_i + 8.3 mM ATP gives almost the same rate of inactivation by NBD-Cl as with 8.3 mM ATP alone, although the observed rate is larger than with 2.5 mM P_i alone. These results indicate that ATP can protect F_1 against inactivation by NBD-Cl, though not as well as P_i, and that ADP not only does not protect F_1 against inactivation by NBD-Cl but also prevents P_i from protecting F_1 effectively.

Correlation between the extent of [^{14}C]NBD-labeling with the ATPase

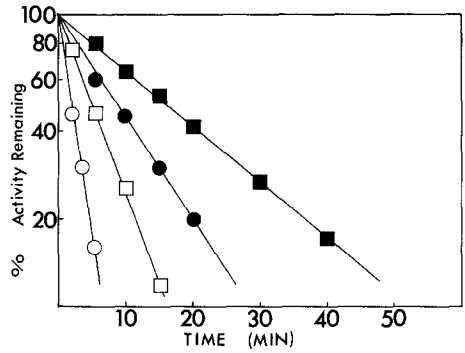

Fig. 3. The effect of sulfate, arsenate, and phosphate on rates of inactivation of F_1 by NBD-Cl. The reaction conditions were the same as Fig. 1, except that the concentrations of sulfate (□), arsenate (●), and phosphate (■) respectively are all 50 mM and the concentration of NBD-Cl is 98 μM. Control values are represented by O.

activity of labeled F_1 is shown in the insert of Fig. 4, in which the percent ATPase activity remaining is plotted against molar ratio of [^{14}C]NBD-label to F_1. The data show that full inactivation of F_1-ATPase by NBD-Cl is accomplished by the binding of 1 mole [^{14}C]NBD-label per mole of F_1, and that after the F_1 has been fully inactivated, more [^{14}C]NBD-label can be attached to F_1. These results are consistent with the published spectrophotometric data of NBD-labeling [14, 26] as well as the [^{14}C]NBD-labeling data [27]. The bound [^{14}C]NBD-label can be removed with DTT with concomi-

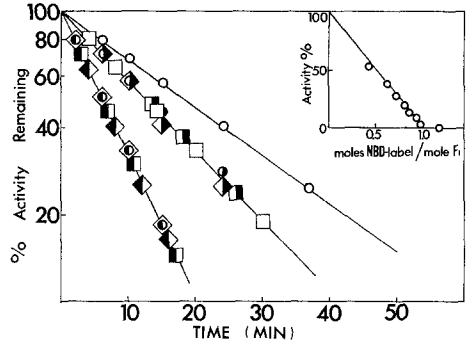

Fig. 4. The effect of ADP and ATP on the inactivation of F_1 by NBD-Cl in the absence and presence of P_i. The reaction conditions were the same as Fig. 1 except that the concentration of NBD-Cl is 49 μM. Notation: ◇, control experiment; □, O, preincubation of F_1 with 1 mM and 2.5 mM P_i respectively; ◆, ◆, preincubation with 8.3 mM ADP and ATP respectively; ◧, ◧, preincubation with 1 mM P_i + 8.3 mM ADP and with 1 mM P_i + 8.3 mM ATP respectively; ◐, ◑, preincubation with 2.5 mM P_i + 8.3 mM ADP and with 2.5 mM P_i + 8.3 mM ATP respectively. In the insert, the percent ATPase activity remaining is plotted against the moles of [^{14}C]NBD-label per mole of ATPase F_1. The procedure is described under Materials and Methods.

tant recovery of ATPase activity provided that the amount of bound label is less than 1 mole per mole of F_1. Kinetic analysis of the radioactive data also show that P_i, ATP, and ADP affect the rate of labeling of F_1 by [^{14}C]NBD-Cl in exactly the same way as these reagents affect the rate of inactivation of the ATPase activity.

The prevention of P_i from protecting F_1 by increasing concentrations of ADP is shown in Fig. 5. If it can be assumed that under the experimental conditions, the conformation of F_1 in aqueous solution is such that ADP and P_i cannot be simultaneously bound at the same active site, the results may be understandable quantitatively by treating ADP as a competitor of P_i for the same site. Inasmuch as ADP by itself does not affect the labeling of F_1 by NBD-Cl, the apparent first-order rate constant k' in the presence of both ADP and P_i is given by

$$k' = k_1\left(\frac{1 + [\mathrm{ADP}]/K_{\mathrm{ADP}}}{1 + [\mathrm{P_i}]/K_{\mathrm{P}} + [\mathrm{ADP}]/K_{\mathrm{ADP}}}\right) + k_2\left(\frac{[\mathrm{P_i}]/K_{\mathrm{P}}}{1 + [\mathrm{P_i}]/K_{\mathrm{P}} + [\mathrm{ADP}]/K_{\mathrm{ADP}}}\right) \tag{3}$$

where K_{ADP} represents the dissociation constant for ADP bound at this site in the F_1–ADP complex. Equation (3) may be rearranged as

$$(k_1 - k')[\mathrm{ADP}]/K_{\mathrm{ADP}} = k'(1 + [\mathrm{P_i}]/K_P) - (k_1 + k_2[\mathrm{P_i}]/K_{\mathrm{P}}) \tag{4}$$

The observed values of $(k_1 - k')[ADP]$ are plotted versus those of the right-hand side in the insert of Fig. 5. The slope gives $K_{ADP} = 210\ \mu M$. Slater et al. [10] reported an estimated value of K_{ADP} 140 μM for the Type III and Type IV binding sites for ADP.

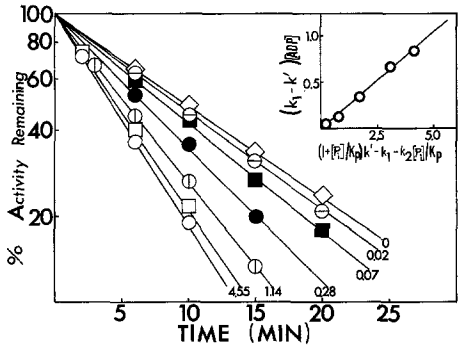

Fig. 5. The effect of different concentrations of ADP on the protection of F_1 by P_i against inactivation by NBD-Cl. Concentrations of ADP in mM are indicated in the figure. The $[P_i]$ during preincubation is 1.96 mM. Other reaction conditions were the same as Fig. 2. The insert shows the determination of the dissociation constant K_{ADP} of bound ADP at the site of F_1. The slope of this linear plot gives $K_{ADP} = 210\ \mu M$.

The effects of nucleotide analogues and their Mg^{2+} complexes on the inactivation of F_1 by NBD-Cl in the absence and presence of P_i are summarized in Table 1. Like ADP, Mg-ADP and AMPCP do not protect F_1 against inactivation by NBD-Cl. ADPCP does protect to almost the same extent as ATP. Mg-AMPCP has some protective effect, but smaller than that of ATP. Mg-ADPCP has a greater protective effect than ATP. Like ADP and ATP, Mg-ADP, AMPCP, ADPCP, and Mg-ADPCP can effectively compete with P_i and prevent the P_i from protecting F_1 against inactivation by NBD-Cl. The exact reason why Mg-AMPCP has a greater protective effect in the presence of P_i than that in the absence of P_i is unknown.

Inactivation of F_1 by Phenylglyoxal

The rates of inactivation of the ATPase activity of F_1 by different concentrations of phenylglyoxal are shown in Fig. 6. The linear plot of log $t_{1/2}$

Table I. Effect of Nucleotide Analogues and Mg^{2+} Complexes on the Inactivation of F_1 by NBD-Cl and Phenylglyoxal respectively in the Absence or Presence of P_i[a]

Labeling reagent	$[P_i]$ (mM)	Nucleotide or complex[c]	$t_{1/2}$ (min)
NBD-Cl[a]	0	(none)	4.6
NBD-Cl[a]	0	ADP	4.6
NBD-Cl[a]	0	ATP	8.2
NBD-Cl[a]	0	AMPCP	4.6
NBD-Cl[a]	0	ADPCP	8.2
NBD-Cl[a]	0	MgADP	4.6
NBD-Cl[a]	0	MgAMPCP	6.5
NBD-Cl[a]	0	MgADPCP	15.0
NBD-Cl[a]	1.0	ADP	4.6
NBD-Cl[a]	1.0	ATP	8.2
NBD-Cl[a]	1.0	AMPCP	4.6
NBD-Cl[a]	1.0	ADPCP	8.2
NBD-Cl[a]	1.0	MgADP	4.6
NBD-Cl[a]	1.0	MgAMPCP	15.0
NBD-Cl[a]	1.0	MgADPCP	15.0
Phenylglyoxal[b]	0	(none)	8.3
Phenylglyoxal[b]	0	ADP	29.5
Phenylglyoxal[b]	0	ATP	29.5
Phenylglyoxal[b]	0	AMPCP	10.5
Phenylglyoxal[b]	0	ADPCP	16.5
Phenylglyoxal[b]	0	MgADP	16.5
Phenylglyoxal[b]	0	MgAMPCP	10.5
Phenylglyoxal[b]	0	MgADPCP	16.5
Phenylglyoxal[b]	1.0	ADP	29.5
Phenylglyoxal[b]	1.0	ATP	29.5

[a]Other experimental conditions are the same as those for Fig. 2.
[b]Other experimental conditions are the same as those for Fig. 6.
[c]Concentration of the adenine nucleotide or its analogue or the Mg^{2+} complex, if present, was 4.54 mM.

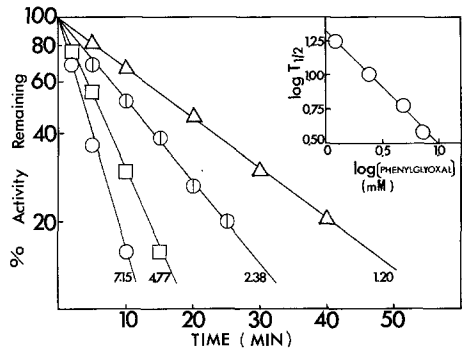

Fig. 6. Inactivation of F_1-ATPase by phenylglyoxal. The reaction conditions were: 50 mM Hepes at pH 8.0, 25 mM NaCl, 2 mM EDTA, 25% glycerol, and 1.2 μM F_1 at 30°C. Concentrations of phenylglyoxal in mM are indicated in the figure. The insert gives the order of the reaction with respect to phenylglyoxal.

of this pseudo-first order reaction versus log [phenylglyoxal] gives a slope of 0.94. Consequently, this inactivation reaction is first order with respect to the concentration of phenylglyoxal and hence can be treated kinetically in the same way as the inactivation of F_1 by NBD-Cl according to Eq. (2).

The effect of different concentrations of P_i on the inactivation of F_1 by a large excess of phenylglyoxal is shown in Fig. 7. The observed continual decrease in inactivation rate as $[P_i]$ is raised suggests that P_i can also protect F_1 against inactivation by phenylglyoxal. The results of kinetic treatment, illustrated in the insert in Fig. 7, gives the following experimental values: $K_p = 1.4$ mM, $k_1 = 2.2 \times 10^{-2}$ mM^{-1} min^{-1}, $k_2 = 1.1 \times 10^{-2}$ mM^{-1} min^{-1} at pH 8.0 and 30°C. Within experimental uncertainties, this value of K_p is in

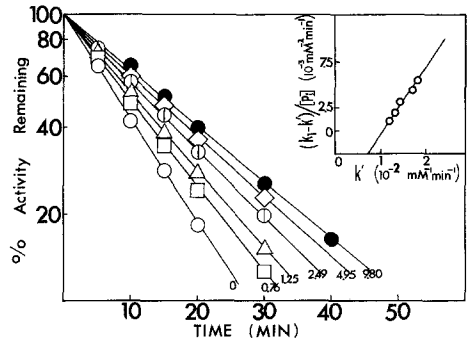

Fig. 7. Effect of different concentrations of P_i on the inactivation of F_1 by phenylglyoxal. The reaction conditions were the same as in Fig. 6 except that the concentration of phenylglyoxal is 3.7 mM. Concentrations of P_i in mM are indicated in the figure. The insert shows the determination of the dissociation constant K_P for P_i bound at the phenylglyoxal-sensitive site of F_1. The slope of this plot gives a value of 1.40 mM for K_P.

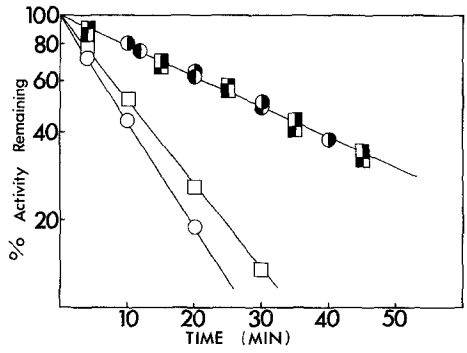

Fig. 8. Effect of ADP and ATP on inactivation of F_1 by phenylglyoxal in the absence or presence of P_i. The reaction conditions were the same as Fig. 7. Notation: ○, control experiment; □, preincubation of F_1 with 1 mM P_i; ◐ , ◑ , preincubation with 4.5 mM ADP and ATP, respectively; ◧ , ◨ , preincubation with 1 mM P_i + 4.5 mM ADP and 1 mM P_i + 4.5 mM ATP, respectively.

agreement with that derived from Fig. 2. The relative values of k_1 and k_2 show that P_i is less effective in protecting F_1 against the inactivation by phenylglyoxal than by NBD-Cl.

The effect of ADP and ATP on the inactivation of F_1 by phenylglyoxal in the absence or presence of P_i is shown in Fig. 8. The rate of inactivation by phenylglyoxal is decreased by 4.5 mM ADP and ATP respectively to almost the same extent, which is not influenced by the presence or absence of 1 mM P_i.

The observed protective effect of different concentrations of ADP on the inactivation of F_1 by phenylglyoxal is summarized in Fig. 9. The effect of

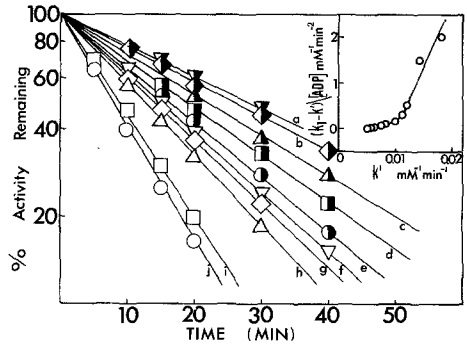

Fig. 9. Effect of different concentrations of ADP on the inactivation of F_1 by phenylglyoxal. The reaction conditions were the same as Fig. 6. Concentrations of ADP are (a) 1.14 mM, (b) 0.57 mM, (c) 0.28 mM, (d) 0.14 mM, (e) 71 μM, (f) 36 μM, (g) 18 μM, (h) 4.4 μM, (i) 1.11 μM, and (j) 0 μM. The insert shows a plot according to Eq. (2) for determining the values of K_{ADP} and k_2.

nucleotide analogues and their Mg^{2+} complexes on the inactivation of F_1 by phenylglyoxal is also summarized in Table I.

It has been shown that the labeling of F_1 by phenylglyoxal [17–19] is not specific for one or two arginine residues. The data in Fig. 9 show that phenylglyoxal can be bound to at least two ADP binding sites with different dissociation constants. The experimentally determined values of $K_{ADP\ 1}$ and $K_{ADP\ 2}$ are quite close to those for the Type I and Type III binding sites respectively reported by Slater [10].

Effect of NBD-Cl on the Inactivation of F_1 by Phenylglyoxal

Since both NBD-Cl and phenylglyoxal inactivate the ATPase via covalent labeling of F_1 at the catalytic site, it would be of interest to know whether modification by one reagent affects the subsequent modification by the other. For this reason, the reaction of F_1 and NBD-labeled F_1 with phenylglyoxal at pH 8.0 has been studied. Aliquots of the incubation mixture were removed at different time intervals and were assayed for ATPase activity in the presence of 2 mM DTT which removed the NBD-label on the active tyrosine residue in F_1. The observed extent of inactivation that is due to the inactivation by phenylglyoxal was followed as functions of time. In both P_i–EDTA–glycerol buffer and triethanolamine–EDTA–glycerol buffer, the inactivation of NBD-F_1 by phenylglyoxal is faster than that of F_1 (Fig. 10).

Discussion

The observation that a tyrosine residue and an arginine residue of F_1 can be labeled by NBD-Cl and phenylglyoxal respectively with concomitant loss

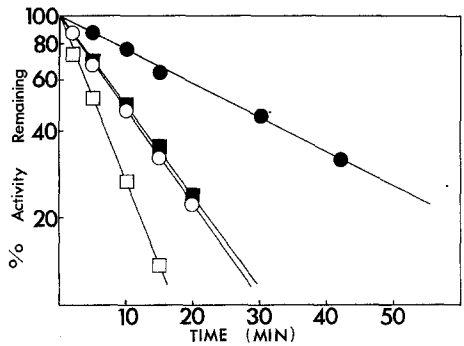

Fig. 10. Effect of NBD-Cl on the labeling of F_1 by phenylglyoxal. The concentration of phenylglyoxal was 3.9 mM. The reaction was performed in a buffer containing 50 mM phosphate or triethanolamine hydrochloride, 2 mM EDTA, 25% glycerol, pH 8.0. Notation: ●, ■, phenylglyoxal inactivation of F_1 and NBD-F_1 respectively in phosphate buffer; ○, □, phenylglyoxal inactivation of F_1 and NBD-F_1 respectively in triethanolamine buffer.

of ATPase activity does not necessarily indicate the presence of an essential phenolic group and an essential guanidinium group at the catalytic site. However, the present data on the protection of F_1 by P_i and ATP respectively against inactivation by NBD-Cl suggest that the labeled essential phenolic group is probably indeed at the catalytic site, since the kinetically deduced values of the dissociation constants $K_P = 1.3$ mM and $K_{ADP} = 210$ μM are quite close to the corresponding values for the catalytic site reported by Slater et al. [10].

The experimentally determined rate constants of the NBD-labeling reaction, $k_1 = 3.5$ mM^{-1} min^{-1} for free F_1 and $k_2 = 0.27$, 3.5, and ~1.8 mM^{-1} min^{-1} for F_1 liganded with P_i, ADP, and ATP respectively at the catalytic site, show that P_i is more effective in protecting this active phenolic group than ATP, and that ADP, which causes the largest protein conformation change [14, 15], has no effect at all. The simplest explanation of this finding is that this active phenolic group is closest to the bound P_i, fairly close to the γ-phosphate group of the bound ATP, but relatively farther away from the adenosine as well as the α- and β-phosphate groups of the bound ADP.

Esch and Allison [20] found that the labeling of F_1 by *p*-fluorosulfonylbenzoyl-5′-adenosine exhibited a different pH dependence from the labeling by NBD-Cl reported by Ferguson et al. [14, 15], and raised the question on the location of the tyrosine residue labeled in the latter case. However, the observed proportionality between inactivation rate and NBD-Cl concentration shows that in this case the activation step involves the direct encounter of the free enzyme with NBD-Cl in solution. Consequently the pH dependence of this labeling reaction should reflect the pK_a of this active phenolic group in the free enzyme. The catalytic site of F_1 should be quite hydrophilic since it binds either ATP + H_2O or ADP + P_i. Thus the continual increase in the rate of labeling of NBD-Cl above pH 9 observed by Ferguson et al. [14] is quite consistent with the expected pK_a of a tyrosine phenolic group in a hydrophilic environment. On the other hand, when this phenolic group reacts with a fairly hydrophobic affinity-labeling reagent like *p*-fluorosulfonylbenzoyl-5′-adenosine, the pH dependence could be quite different. If the reaction shows nonlinear concentration dependence, the observed pH dependence of labeling rate would be very difficult to interpret without extensive additional kinetic data because of possible change in rate-limiting step, interactions in the protein–reagent complex, etc.

Because of the additional nonspecific labeling of arginine residues in F_1 by phenylglyoxal [16, 19], the corresponding inactivation data have to be interpreted with caution. The data from Figs. 6 to 10 show that modification of the essential guanidinium group of Arg can be protected by ADP, ATP, and P_i respectively. The experimental dissociation constants deduced from these data, $K_{ADP} = 160$ μM and $K_p = 1.4$ mM, are in approximate agreement with the corresponding values deduced from the NBD-Cl data. Consequently

we may conclude that this essential guanidinium group is at the same catalytic site where the active phenolic group of the essential tyrosine residue is located.

The experimental rate constants of the phenylglyoxal-labeling reaction, $k_1 = 0.022\ mM^{-1}\ min^{-1}$ for free F_1 and $k_2 = 0.011$ and $0.005\ mM^{-1}\ min^{-1}$ for F_1 liganded by P_i and ADP respectively at the catalytic site, show that ADP and ATP (Fig. 8) are more effective than P_i in protecting this essential guanidinium group. Here again the simplest interpretation is that this positively charged guanidinium group is closer to the α- and β-phosphate groups of the bound ADP or ATP than to the bound P_i.

The data in Fig. 10 are interesting in demonstrating that the NBD-label on the active phenolic group at the catalytic site of F_1 actually facilitates subsequent phenylglyoxal labeling of the neighboring guanidinium group. The possibility that the NBD-label could enhance the hydrophobicity and hence increase the affinity of the catalytic site for phenylglyoxal deserves further investigation.

Acknowledgments

This work was supported in part by research grants from the National Institute of General Medical Sciences (GM19990) and the National Science Foundation (PCM-7715002). We thank Ms. Betty Stone and Judith Olsen for the preparation of mitochondria coupling factor F_1.

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