Biochimica et Biophysica Acta, 679 (1982) (1982) 269–278
Elsevier Biomedical Press

BBA 41037

PYRIDOXYLATION OF ESSENTIAL LYSINE RESIDUES OF MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE

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(Received July 6th, 1981)

Key words: F_1-ATPase; Pyridoxal phosphate; Lysine residue; (Bovine heart)

1. Soluble beef-heart mitochondrial ATPase (F_1) was incubated with [^{3}H]pyridoxal 5′-phosphate and the Schiff-base complex formed was reduced with sodium borohydride. Spectral measurements indicate that lysine residues are modified and gel electrophoresis in the presence of detergent shows the tritium label to be associated with the two largest subunits, α and β. 2. In the absence of protecting ligands, the loss of ATP hydrolysis activity is linearly dependent on the level of pyridoxylation with complete inactivation correlating to 10 mol pyridoxamine phosphate incorporated per mol enzyme. Partial inactivation of F_1 with pyridoxal phosphate has no effect on either the K_m for ATP or the ability of bicarbonate to stimulate residual hydrolysis activity, suggesting a mixed population of fully active and fully inactive enzyme. 3. In the presence of excess magnesium, the addition of ADP or ATP, but not AMP, decreases the rate and extent of modification of F_1 by pyridoxal phosphate. The non-hydrolyzable ATP analog, 5′-adenylyl-β,γ-imidodiphosphate, is particularly effective in protecting F_1 against both modification and inactivation. Efrapeptin and P_i have no effect on the modification reaction. 4. Prior modification of F_1 with pyridoxal phosphate decreases the number of exchangeable nucleotide binding sites by one. However, pyridoxylation of F_1 is ineffective in displacing endogenous nucleotides bound at non-catalytic sites and does not affect the stoichiometry of P_i binding. 5. The ability of nucleotides to protect against modification and inactivation by pyridoxal phosphate and the loss of one exchangeable nucleotide site with the pyridoxylation of F_1 suggest the presence of a positively charged lysine residue at the catalytic site of an enzyme that binds two negatively charged substrates.

Introduction

The terminal coupling factor, F_1, catalyzes the synthesis of ATP during mitochondrial oxidative phosphorylation. The enzyme consists of five different subunits, designated α, β, γ, δ and ϵ in order of increasing mobility on sodium dodecyl sulfate polyacrylamide gels [1]. Modifications of F_1 with photoaffinity [2] and alkylating [3,4] analogs of adenine nucleotides have provided evidence that the β subunit contains the catalytic site. There appear to be three copies of this subunit per F_1 [5–8].

Modification studies have shown the presence of essential arginine [9,10], carboxyl [11,12], and tyrosine [4,13] residues on the enzyme. In a previous

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Abbreviations used: F_1, in the isolated soluble form used in these studies, the mitochondrial enzyme is referred to as an ATPase. Efrapeptin, also referred to in the literature as efrastatin and A23871; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Ado*PP*[NH]*P*, 5′-adenylyl-β,γ-imidodiphosphate; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl.

study, we provided evidence that F_1 contains a single essential arginine residue [14]. Efrapeptin, a low-molecular-weight peptide antibiotic that inhibits F_1, protects the essential residue from modification by phenylglyoxal. Combined with evidence that efrapeptin and P_i binding are mutually exclusive [15,16], these results suggest the presence of a positively charged arginine residue at the binding site for $H_2PO_4^-$ [17]. The enzyme also binds a second negatively charged substrate, $MgADP^-$, and in the experiments reported here we have investigated the possibility that a positively charged lysine residue participates at this binding site.

Pyridoxal 5'-phosphate has been found to form a Schiff-base complex with lysine residues on a variety of enzymes, including many that have no requirement for pyridoxal phosphate as a cofactor [18]. A common feature of enzymes that are inactivated by pyridoxal phosphate is that they contain allosteric or active sites that normally bind sugar phosphates [19] or sugar-phosphate-containing cofactors such as adenine nucleotides [20,21]. The modification reaction appears to be highly specific [22], and borohydride reduction to a stable derivative [23] has been used to characterize amino acid residues present at substrate binding sites [24]. In a general survey utilizing amino-acid-specific reagents, Ferguson et al. [25] reported that pyridoxal phosphate inhibits F_1. The amount of inactivation decreased with the addition of ADP or ATP. Godinot et al. [26] have confirmed this observation and have noted pyridoxal phosphate-mediated photoinactivation of F_1. In the present study, the effects of various ligands on modification of F_1 by [^{3}H]pyridoxal phosphate and the subunit distribution of incorporated label are described. The effects of pyridoxylation of F_1 on adenine nucleotide and P_i binding sites and on bicarbonate stimulation of ATP hydrolysis are also presented.

Experimental procedures

Materials

ATP, ADP, AMP, NADH, $NaBH_4$, phospho*enol*-pyruvate, pyridoxal 5'-phosphate, 1-fluoro-2,4-dinitrobenzene (FDNB) and Hepes were obtained from Sigma Chemical Co. Solutions of pyruvate kinase and lactate dehydrogenase in 50% glycerol were purchased from Boehringer-Mannheim Biochemicals and stored at −20°C. HPLC-purified disodium 5'-adenylyl-β,γ-imidodiphosphate (Ado*PP*[NH]*P*), [^{3}H]Ado*PP*[NH]*P*, $^{32}P_i$ and [^{14}C]ATP were obtained from ICN and [^{3}H]$NaBH_4$ (5 Ci/mmol) from Amersham Corp. Efrapeptin was a gift from Dr. R.L. Hamill of Eli Lilly Co.

Synthesis of [^{3}H]pyridoxal 5'-phosphate

^{3}H-labeled pyridoxal phosphate was prepared by the reduction of pyridoxal phosphate with [^{3}H]-$NaBH_4$ followed by reoxidation according to the method of Stock et al. [27] as modified [28,29]. In order to minimize gas formation during the final chromatography step, the pH of the preparation was adjusted to 6.5 by addition of KOH instead of $KHCO_3$. From 25 mg starting material, 3.85 mg final product were obtained with a specific activity of $5.44 \cdot 10^5$ cpm/nmol. The lyophilized product was dissolved in water to a final concentration of 7.7 mM and stored in a foil-wrapped tube at −70°C. Under these conditions, pyridoxal phosphate is stable for several months [30]. Prior to use, an aliquot of [^{3}H]pyridoxal phosphate was diluted to 10- to 20-fold with carrier pyridoxal phosphate. A small sample of this stock solution was diluted with 0.1 M NaOH, and the concentration was determined spectrophotometrically at 388 nm using a molar extinction coefficient of $6.6 \cdot 10^3$ [30].

Preparation of F_1

Heavy beef-heart mitochondria were prepared by the method of Smith [31] and stored at −70°C. F_1 was isolated by the procedure of Knowles and Penefsky [1]. The purity of the enzyme preparation was determined by using both dissociating and non-dissociating polyacrylamide gel electrophoresis. F_1 was stored as an ammonium sulfate precipitate at 4°C.

Immediately prior to use, F_1 was collected by centrifugation in a Beckman airfuge and dissolved in 0.25 M sucrose/10 mM Hepes (pH 8.0)/2 mM EDTA/4 mM ATP at room temperature. Aliquots of 100 μl each were de-salted on 1.0 ml Sephadex centrifuge columns [32] equilibrated with 0.15 M sucrose/10 mM Hepes (pH 8.0)/0.1 mM EDTA/0.5 mM ATP. This buffer was also present in the modification reactions described below, since the volume of F_1 added to the reaction mixture constituted 80% or more of the total volume. During long incubations, the inclu-

sion of ATP and sucrose in the buffer of control samples lacking pyridoxal phosphate was found effective in protecting F_1 from aggregation and degradation as measured by non-dissociating gel electrophoresis and ATP hydrolysis assays. However, the pyridoxylation of F_1 was not affected by the low concentration of ATP used in the buffer.

In experiments designed to determined the effect of pyridoxylation of F_1 on the retention of nucleotides that are tightly-bound at non-catalytic sites, F_1 was depleted of endogenous nucleotides by gel-filtration chromatography in the presence of 50% glycerol [33]. Nucleotide-depleted F_1 was reconstsituted by a rapid 12-fold dilution of the glycerol with buffer containing magnesium and [^{14}C]ATP. ^{14}C-labeled nucleotide was then displaced from exchangeable sites by incubation for 2 min with 1 mM non-radioactive MgATP as described [8]. With passage through a Sephadex centrifuge column, the enzyme retains three ^{14}C-labeled nucleotides at non-catalytic sites.

[^{3}H]Pyridoxylation of F_1

Reaction mixtures were adjusted to a final volume of 480 μl unless noted otherwise and the reaction was initiated by the addition of [^{3}H]pyridoxal phosphate. The concentration of F_1, pyridoxal phosphate, and other additions are given in the figure captions. Reactions were carried out under low-light conditions [34] at room temperature. At the times indicated, 95 μl aliquots were removed and the reaction stopped by mixing with 10 μl freshly prepared 1.0 M $NaBH_4$/ 0.1 M pyridoxine 5′-phosphate. Following a 5–15 s incubation, the samples were applied to Sephadex centrifuge columns equilibrated with 0.15 M sucrose/ 10 mM Hepes (pH 8.0)/0.1 mM EDTA/ and 0.5 mM ATP. The column effluents were diluted with 500 μl of the same buffer and aliquots were removed for assays of protein concentration, ATP hydrolysis activity and tritium incorporation. In other experiments where non-radioactive pyridoxal phosphate was used, variations in the procedure are noted in the figure and table captions.

An incubation of 5 s combined with the time required to separate enzyme from borohydride on the centrifuge column appears to be sufficient for reduction of the Schiff base which forms between pyridoxal phosphate and the enzyme. Upon addition of borohydride the yellow color of medium pyridoxal phosphate was bleached almost immediately and no differences in amount of radioactivity incorporated into the protein fraction could be detected with incubations ranging from 5 s to 15 min. The shorter times were used since prolonged incubation with sodium borohydride caused partial inactivation of F_1 even in the absence of pyridoxal phosphate. Inactivation of F_1 by pyridoxal phosphate is nearly completely reversed upon removal of unbound reagent if the borohydride reduction step is omitted.

Control samples were incubated under identical conditions except for the omission of pyridoxal phosphate. In experiments where tritium incorporation was measured, an appropriate amount of [^{3}H]pyridoxal phosphate was added to the borohydride solution 15 s prior to mixing with an aliquot from the incubation mixture. The inclusion of a large excess of reduced, unlabeled pyridoxine phosphate in the borohydride solution decreased the radioactivity in column effluents of control samples. Indeed, in most cases the controls contained less than 50 cpm and were indistinguishable from the column effluents of blanks that contained [^{3}H]pyridoxal phosphate but lacked F_1.

Other methods

Protein was determined by the Hartree [35] modification of the method of Lowry et al. [57]. Quantitative precipitation of protein by the method of Bensadoun and Weinstein [36] was carried out prior to assay in order to remove interfering substances. Defatted bovine serum albumin was used as a standard. The amount of protein determined by this assay was divided by 1.18 to convert to dry weight of F_1 [37] and a molecular weight of 347 000 was used to calculate F_1 concentration [38].

ATP hydrolysis activity was measured at pH 7.8 and 30°C by monitoring the decrease in absorbance at 340 nm using an NADH-linked, ATP-regenerating system. The assay mixture contained 2 mM $MgCl_2$ and 500 μM ATP. Other components were present at the concentrations described [39] except for the omission of uncoupler and cyanide. Assays were run for a time sufficient to obtain accurate steady-state rates.

Electrophoresis on non-dissociating polyacrylamide disc gels was performed as described by Ornstein [40] and Davis [41]. Gels were prepared accord-

ing to the 'Tris' system of Jovin et al. [42], except that the lower gel contained 5% or 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide. Electrophoresis in the presence of sodium dodecyl sulfate was carried out by the method of Weber and Osborn [43]. Following staining with Coomassie brilliant blue G-250, protein bands were scanned on a Beckman 25 spectrophotometer equipped with an automated gel scanner.

Aliquots of 100 μl of the diluted effluents from the Sephadex centrifuge columns were added to 10 ml Instagel (Packard Inst. Co.). In the experiment reported in Fig. 5, gel slices were incubated in 0.5 ml Soluene 100 (Packard Inst. Co.) at 50°C for 2 h with constant agitation. Samples were cooled to room temperature and 10 ml Instagel were added. The tritium content of the samples was measured using a Packard 2425 counter.

The purity and stability of adenine nucleotide solutions was checked by thin-layer chromatography on poly(ethyleneimine)-cellulose plates. The solvent system contained 0.5 M LiCl and 2.0 N formic acid [33].

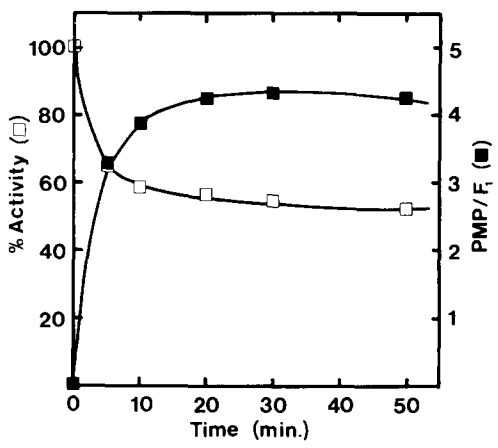

Fig. 1. Time course of pyridoxal phosphate modification and inactivation of F_1. F_1 was incubated at 0.92 mg per ml with 0.56 mM [^{3}H]pyridoxal phosphate/0.15 M sucrose/10 mM Hepes (pH 8.0)/0.1 mM EDTA/0.5 mM ATP, in a final volume of 480 μl. At the times indicated, aliquots were removed and reaction was stopped by addition of borohydride. F_1 was assayed for ATP hydrolysis activity (□) and ^{3}H-incorporation (■). The hydrolysis rate of a control sample lacking pyridoxal phosphate was 34 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (100%).

Results

The effects of incubation time, pyridoxal phosphate concentration, and Mg^{2+} on modification and inactivation of F_1

F_1 was incubated with 0.56 mM [^{3}H]pyridoxal phosphate, the reaction stopped by addition of $NaBH_4$, and unbound ligands removed as described under Experimental procedures. The modified enzyme was assayed for covalently bound [^{3}H]pyridoxamine 5'-phosphate (PMP) and ATP hydrolysis activity. As shown in Fig. 1, the modification reaction reaches equilibrium in about 20 min.

Fig. 2 shows the modification and inactivation obtained when the concentration of [^{3}H]pyridoxal phosphate is varied between 0.15 mM and 3.0 mM and incubation is continued for 20 min. The amount of covalently-bound pyridoxamine phosphate was determined by ^{3}H-incorporation and confirmed spectrophotometrically at 326 nm using a buffer of pH 7.0 and an estimated molar extinction coefficient of 5850 for pyridoxal-lysine moieties [44]. Activity of the pyridoxylated enzyme was calculated relative to a control sample incubated in the absence of pyridoxal phosphate. Even at pyridoxal phosphate concentrations higher than those shown in Fig. 2, a residual, efrapeptin-sensitive ATP hydrolysis activity of 5–10% of the control rate was always observed. This residual activity may reflect the equilibrium between bound pyridoxal phosphate and the Schiff-base complex at the time of addition of borohydride.

F_1 samples, modified by up to 5.3 mol pyridoxamine phosphate per mol enzyme, were subjected to electrophoretic analysis on non-dissociating 5% polyacrylamide disc gels. In each case the F_1 band was symmetrical. The relative mobility of the F_1 band increased with increase in extent of modification, consistent with an increase in negative charge (data not shown).

The modification of other enzymes by pyridoxal phosphate has been found to require [45] or to be facilitated by [46] the addition of magnesium. In Fig. 3 the addition of magnesium is shown to increase both the rate and extent of pyridoxal phosphate-mediated inactivation of ATP hydrolysis activity. A similar increase in the rate and extent of modification is also found. In Fig. 4 the remaining ATP hydrolysis activity is plotted against mol pyridoxa-

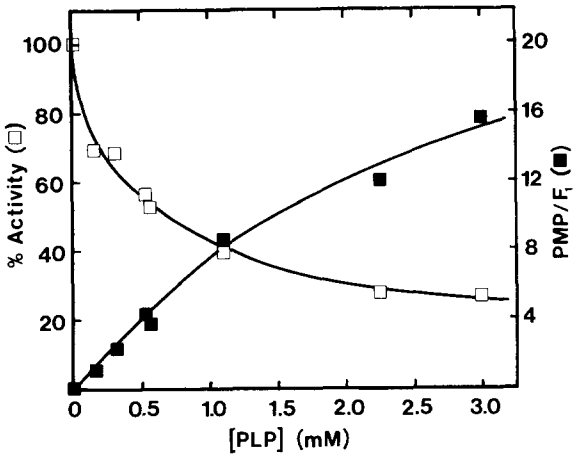

Fig. 2. Effect of pyridoxal phosphate concentration ([PLP]) on modification and inactivation of F_1. F_1 was incubated for 20 min with concentrations of [^{3}H]pyridoxal phosphate which varied from 0.15 to 3.0 mM. The total volume of each reaction mixture was 100 μl. Reaction was stopped by addition of borohydride and F_1 was assayed for ATP hydrolysis activity (□) and ^{3}H-incorporation (■). The ATP hydrolysis activity of a control sample lacking pyridoxal phosphate was 34 μmol · min^{-1} · mg^{-1} (100%).

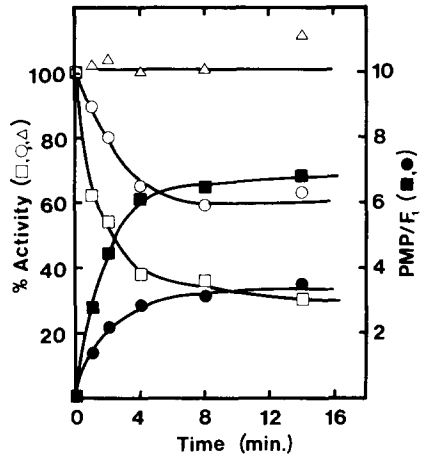

Fig. 3. The effect of magnesium on the modification and inactivation of F_1 by pyridoxal phosphate. Incubations contained 1.2 mg F_1 per ml and 0.79 mM [^{3}H]pyridoxal phosphate with (□, ■) or without (○, ●) 9.1 mM $MgCl_2$. Samples were removed at the times indicated. F_1 was assayed for ATP hydrolysis activity (□, ○) and ^{3}H-incorporation (■, ●). The ATP hydrolysis activity of a control sample containing $MgCl_2$ but not pyridoxal phosphate (▲) averaged 37 μmol · min^{-1} · mg^{-1} (100%).

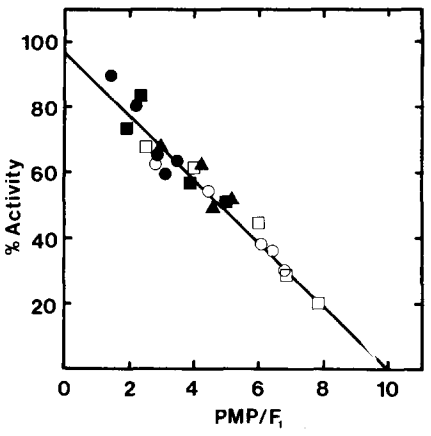

Fig. 4. Correlation of loss of ATP hydrolysis activity with the amount of pyridoxamine-phosphate incorporated. F_1 was pyridoxylated in the presence (○, □) or absence (●, ■, ▲) of 9.1 mM $MgCl_2$ as described for Fig. 3. Different sets of symbols represent separate experiments. The line drawn is a linear least-squares best fit of the data points.

mine phosphate incorporated per mol F_1. Data points obtained with or without added magnesium fall on a straight line extrapolating to about 10 mol pyridoxamine phosphate per mol F_1 for complete inactivation. This stoichiometry represents about 5% of the total number of lysine residues [38].

The effect of partial inactivation of F_1 with pyridoxal phosphate on the K_m for ATP

The large titer of pyridoxal moieties required for complete in activation could be due to the presence of a number of lysine residues that are essential for full enzymatic activity but are not located at the catalytic site. Alternatively, there may be a combination of essential and nonessential residues, all with the same reactivity with respect to pyridoxal phosphate modification under the conditions used. In order to help distinguish between these possibilities, the effect of partial inactivation of F_1 on the K_m for ATP was measured. Control enzyme gave a V of 63 μmol · min^{-1} · mg^{-1} and a K_m for ATP of 67 μM. The maximum velocities of modified enzyme were inhibited by 24, 57 and 70% with no experimentally significant change in K_m.

The effects of substrates and a substrate analog on the modification of essential residues of F_1 by pyridoxal phosphate

Preincubation of F_1 with ADP or ATP but not AMP causes a significant decrease in mol pyridoxamine phosphate incorporated per mol F_1 (Table I, experiment 1). There is no difference in the level of protection afforded by ADP or ATP; however, under the conditions used, the ATP was rapidly hydrolyzed to ADP. In order to enhance the possibility of detecting substrate protection of active-site lysine residues, a non-hydrolyzable ATP analog, Ado*PP*[NH]*P*, was also tested. As shown in Table I, experiment 2, increasing concentrations of Ado*PP*[NH]*P* provide increasing protection against both inactivation of F_1 and incorporation of pyridoxal phosphate.

In contrast, the rates of modification and inactivation of F_1 by pyridoxal phosphate in the presence of 5 mM $MgCl_2$ are not affected by the addition of 12 mM phosphate (data not shown). The failure of phosphate to protect essential lysine residues might be related to difficulties in keeping the phosphate site saturated during incubation with pyridoxal phosphate. In view of the demonstration that efrapeptin and P_i binding to F_1 are mutually exclusive [15,16] it was of interest to test the effect of this tight-binding peptide antibiotic on modification by pyridoxal phosphate. Preincubation of F_1 for 20 min with sufficient efrapeptin to inhibit ATP hydrolysis activity by over 98% had no effect on subsequent modification of F_1 by pyridoxal phosphate (data not shown). The effect of efrapeptin on pyridoxal phosphate-dependent inactivation of ATP hydrolysis activity was not tested since efrapeptin inhibits hydrolysis and we have been unable to find conditions that reverse efrapeptin binding to soluble F_1.

The effect of modification of essential lysine residues of F_1 on binding sites for substrates and substrate analogs

The protection afforded by adenine nucleotides (Table I) suggested that pyridoxal phosphate may modify a lysine residue at a nucleotide binding site. This possibility was further examined by measuring the effect of modification on the stoichiometry of adenine nucleotide sites. Mitochondrial F_1 has been shown to bind nucleotide at three exchangeable sites that are distinct from three noncatalytic sites [8].

TABLE I

THE EFFECTS OF ADENINE NUCLEOTIDES ON THE MODIFICATION AND INACTIVATION OF F_1 BY PYRIDOXAL PHOSPHATE

	Incubation conditions [a]	Additions [b]	PMP [c]/F_1	ATP hydrolysis [d] (% of control)
1	1 mg F_1/ml, 0.79 mM [^{3}H]pyridoxal phosphate, 10 mM $MgCl_2$, 14 min	none	4.3	
		3.8 mM AMP	4.1	
		4.1 mM ADP	2.8	
		25 mM ATP	2.8	
2	1 mg F_1/ml, 0.79 mM [^{3}H]pyridoxal phosphate, 0.5 mM $MgCl_2$, 6 min	2.5 μM Ado*PP*[NH]*P*	3.9	60
		5.0 μM Ado*PP*[NH]*P*	3.5	65
		10 μM Ado*PP*[NH]*P*	2.8	74
		83 μM Ado*PP*[NH]*P*	1.2	83

[a] The reaction buffer contained 150 mM sucrose/10 mM Hepes (pH 8.0)/0.1 mM EDTA/0.5 mM ATP.

[b] Prior to addition of [^{3}H]pyridoxal phosphate, F_1 was preincubated with or without adenine nucleotides at the concentrations indicated for 2 min in experiment 1 and for 15 min in experiment 2.

[c] Pyridoxamine phosphate.

[d] Control incubations were prepared containing Ado*PP*[NH]*P* at the concentrations indicated but lacking pyridoxal phosphate. Following removal of unbound nucleotide, small aliquots of the centrifuge column effluents were assayed for ATP hydrolysis activity. Measurement was continued for a time sufficient to ensure dissocation of the protecting ligand and the linear final rates for the controls (40 ± 2 μmol · min^{-1} · mg^{-1}) were indpendent of the concentration of Ado*PP*[NH]*P* used.

When Ado*PP*[NH]*P* is preincubated with F_1, subsequent modification with pyridoxal phosphate results in the displacement of only a small amount of ligand from the exchangeable sites (Table II). This is consistent with the ability of Ado*PP*[NH]*P* to partially protect F_1 against pyridoxal phosphate-mediated inactivation (Table I). However, when F_1 is first modified by pyridoxal phosphate and then incubated with Ado*PP*[NH]*P*, one of the three binding sites for the ligand is lost (Table II).

In order to assess the effect of pyridoxal phosphate on the non-catalytic nucleotide sites, the sites were specifically loaded with ^{14}C-labeled adenine nucleotide as described under Experimental procedures. As shown in Table II, pyridoxylation of F_1 did not result in the displacement of any nucleotide from noncatalytic sites. Prior modification of F_1 with pyridoxal phosphate also had no effect on the stoichiometry of phosphate binding (Table II), consistent with the inability of phosphate to protect F_1 from modification or inactivation.

In a previous study it has been suggested that FDNB modifies a lysine residue at the catalytic site of F_1 [47]. However, other investigators have provided evidence that FDNB inactivation is due to the modification of an essential tyrosine residue not present at the catalytic site [48]. Consistent with the latter view is the finding that inactivation of F_1 by FDNB has no effect on the stoichiometry of exchangeable nucleotide sites regardless of the order of binding and modification steps (Table II).

The effect of pyridoxylation of F_1 on bicarbonate stimulation of ATP hydrolysis

The presence of an anion binding site on beef-heart mitochondrial F_1 has been proposed on the basis of the ability of anions, particularly bicarbonate, to stimulate the rate of ATP hydrolysis [49]. To determine whether a positively charged lysine residue might be present at the anion binding site, the effect

TABLE II

THE EFFECTS OF MODIFICATION OF F_1 BY PYRIDOXAL PHOSPHATE AND FDNB ON BINDING SITES FOR PHOSPHATE AND ADENINE NUCLEOTIDES

F_1 was incubated with either pyridoxal phosphate, followed by borohydride reduction (F_1-pyridoxamine phosphate), or with FDNB (F_1-DNP) under conditions which resulted in loss of 90% or more of the ATP hydrolysis activity in the absence of protecting ligands. Unless otherwise noted, both the modification and binding steps were performed in 0.15 M sucrose/10 mM Hepes (pH 8.0)/0.1 mM EDTA/2.0 mM $MgCl_2$ and unbound reagent or ligand was removed by column centrifugation. The stoichiometry of P_i sites was determined by a Scatchard plot analysis of binding data obtained from incubating control or pyridoxylated F_1 with 10–92 μM P_i for 20 min [32]. Non-catalytic sites were specifically loaded with ^{14}C-labeled nucleotide. To measure the stoichiometry of exchangeable nucleotide sites, F_1 was incubated with 50 to 60 μM Ado*PP*[NH]*P* for 1 h [8]. In experiments where binding to exchangeable sites preceded the modification reaction, unbound Ado*PP*[NH]*P* was not removed until the modification reaction was terminated. In experiments where modification preceded binding, unreacted reagent was removed by column centrifugation prior to addition of ligand.

Type of site	Ligand	Sequence of steps	mol ligand/mol F_1	mol ligand/mol F_1
			Control F_1	F_1-PMP [c]
Exchangeable nucleotide	[^{3}H]Ado*PP*[NH]*P*	Bind/modify	3.0	2.7
		Modify/bind	2.9	2.0
Noncatalytic nucleotide	[^{14}C]ATP	Bind/modify	3.1	3.1
Phosphate	[^{32}P]P_i	Modify [a]/bind [b]	1.2	1.2
			Control F_1	F_1-DNP
Exchangeable nucleotide	[^{3}H]Ado*PP*[NH]*P*	Bind/modify	3.0	3.1
		Modify/bind	3.2	3.3

[a] The buffer used comprised 0.15 M sucrose/10 mM Hepes (pH 8.0)/0.1 mM EDTA/0.5 mM ATP.
[b] The buffer used comprised 0.25 M sucrose/50 mM Tris-HCl (pH 7.5)/2 mM $MgCl_2$.
[c] F_1-pyridoxamine phosphate.

of partial inactivation of F_1 with pyridoxal phosphate on the ability of bicarbonate to stimulate the residual activity was tested. The ability of bicarbonate to stimulate ATP hydrolysis activity by approximately 60% is undiminished, even when the residual activity of pyridoxylated F_1 is only 30% of the control. Assays for ATP hydrolysis using 40–400 μM substrate gave linear double-reciprocal plots in the absence or presence of bicarbonate and bicarbonate was found to have no effect on the K_m for ATP (Cross, R.L., unpublished results).

Subunit distribution of pyridoxylated residues on F_1

Incubation of F_1 for 90 s in the presence of 0.8 mM [^{3}H]pyridoxal phosphate and absence of protecting ligands results in the incorporation of 2.2 mol pyridoxamine phosphate per mol F_1. As shown in Fig. 5, radioactivity is predominantly associated with the α and β subunits which appear to be equally labeled. Trace amounts of tritium label also co-migrate with the γ and ϵ subunits. More extensive pyridoxylation of F_1 does not change the relative distribution of the tritium label.

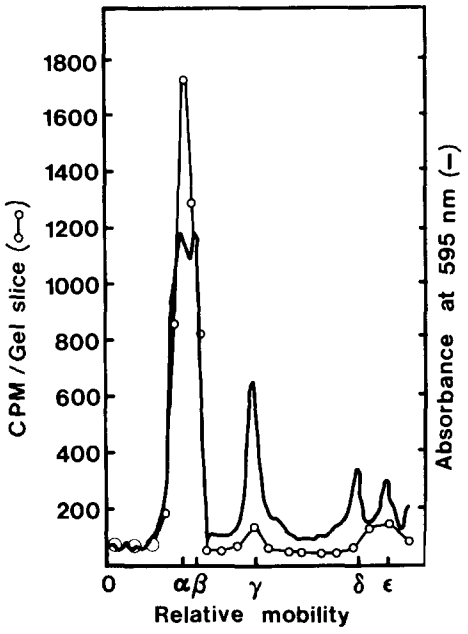

Fig. 5. Subunit distribution of incorporated radioactivity in [^{3}H]pyridoxylated F_1. F_1 at 1.1 mg/ml was incubated with 0.8 mM [^{3}H]pyridoxal phosphate for 90 s. The reaction was stopped, unbound ligand removed, a small amount of carrier F_1 added, and the sample was analyzed on a sodium dodecyl sulfate polyacrylamide gel. The gel was scanned after staining protein bands with Coomassie blue (solid line). The gel was then sliced radioactivity determined as described.

Discussion

Spectral measurements at a wavelength characteristic of pyridoxyl-lysine moieties [44] give values for the mol pyridoxamine phosphate incorporated per mol F_1 that are in agreement with values calculated from tritium incorporation (Fig. 2). In the following discussion the reactive moieties will be referred to as lysine residues; however, a chemical identification has not been obtained.

In view of the large titer required for complete inactivation, it was important to determine whether partial inactivation results in a homogeneous population of enzyme molecules with partial loss of activity or a mixed population of fully active plus fully inactive enzyme. The constant K_m value for ATP at various levels of inhibition of F_1 by pyridoxal phosphate indicates a loss of all or none of the activity. This is to be expected if residues essential for activity are located at the catalytic site. Although there is apparent heterogeneity with regard to loss of enzymatic activity, electrophoretic analysis of pyridoxylated F_1 under nondissociating conditions indicates fairly homogenous labelling. The simplest explanation for these results is that the ten residues modified with complete inactivation of F_1 include both essential and nonessential groups that react with pyridoxal phosphate at the same rate. This explanation is consistent with the linear relationship between the loss of activity and incorporation of reagent (Fig. 4) and could result from a rate limitation in the formation of the Schiff-base complex rather than in the binding of pyridoxal phosphate at more than one type of site.

A substantial amount of evidence has been presented that indicates strong cooperative interactions between the multiple copies of the catalytic subunit on mitochondrial F_1 [49,50,51] (see Ref. 17). Assuming three copies of the catalytic subunit per F_1 [5–8], previous studies have shown phenylglyoxal to be a '1/3-of-the-sites reagent'. This is based on the findings that the modification of a single fast-reacting essential residue leads to complete inactiva-

tion [14] and that inactivation of F_1 by phenylglyoxal results in the loss of one exchangeable Ado*PP*[NH]*P* binding site [8]. Although pyridoxal phosphate does not show the specificity for modification obtained with phenylglyoxal, the correlation between loss of activity and loss of one exchangeable nucleotide binding site (Table II) is similar.

The true substrates for ATP synthesis appear to be the monovalent anions, $H_2PO_4^-$ [15] and $MgADP^-$ [52]. Protection by efrapeptin of a single fast-reacting residue on F_1 from modification by phenylglyoxal [14] and evidence that efrapeptin and phosphate binding are mutually exclusive [15,16] are consistent with the presence of a positively charged arginine moiety at the binding site for $H_2PO_4^-$. The protection of essential lysine residues by adenine nucleotides (Table I) and the effect of pyridoxylation on the number of exchangeable nucleotide sites (Table II) suggests the presence of a positively charged lysine moiety at the binding site for $MgADP^-$. Consistent with a close proximity of an arginine and lysine residue is the finding that prior modification of chloroplast F_1 with an arginyl reagent decreases subsequent incorporation of pyridoxal phosphate [53].

Peters et al. [54] have recently studied the reaction of pyridoxal phosphate with bacterial F_1. Using the extinction coefficient for ϵ-pyridoxyllysine they report complete inactivation of the enzyme with incorporation of 6 mol pyridoxamine phosphate per mol F_1. However, Blackburn and Schachman [44] have shown that the extinction coefficient for pyridoxylated lysine residues of polypeptide chains is considerably less than that for pyridoxylated lysine. Using the latter extinction coefficient, the data of Peters et al. [54] give a ratio of ten pyridoxamine phosphate per bacterial F_1 at complete inhibition as found for the mitochondrial enzyme (Fig. 4). It was initially reported that chloroplast F_1 contains a large number of essential amino groups that are modified by pyridoxal phosphate [53]. Subsequent studies defined conditions that allow complete inactivation with the modification of only one α and one β subunit per enzyme [45]. In studies of a member of a different class of membrane-bound ATPase, Murphy [55] has reported the pyridoxylation of a single essential lysine residue on the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum.

Unless conditions can be defined that increase the specificity of the reaction between pyridoxal phosphate and the mitochondrial enzyme, this reagent holds little promise for characterizing amino acid sequences in the vicinity of the active site. An initial report on the modification of F_1 with FDNB suggested that this reagent might provide a more specific probe of active site lysine residues [47]. However, a subsequent study by Andrews and Allison [48] indicated that FDNB reacts with the same essential tyrosine residue that can be modified by Nbf-Cl and which is not located at the catalytic site [6,50]. In assessing the relationship between essential amino acid residues and the catalytic site of F_1, Ado*PP*[NH]*P* would appear to be a particularly useful probe [8]. The binding of nucleotide at exchangeable sites is generally believed to reflect substrate binding at catalytic sites (see Ref. 56) and Ado*PP*[NH]*P* would presumably overlap both the ADP and P_i binding domains. Consistent with the conclusions of Andrews and Allison, we find that neither FDNB (Table II) nor Nbf-Cl [8] affects the stoichiometry of Ado*PP*[NH]*P* binding at exchangeable nucleotide sites.

The possible presence of a lysine residue at the binding site on F_1 for activating anions was also considered. Partial inactivation of F_1 with pyridoxal phosphate, however, has no effect on bicarbonate stimulation of the residual activity. Although this does not exclude the presence of a lysine residue at the anion site, it would appear to rule out the existence of an anion site that can be modified by pyridoxal phosphate without appreciable loss of enzymatic activity.

Acknowledgements

We wish to thank Mr. Thomas Barra, Mrs. Connie Clark and Miss Wendy Wessels for their excellent technical assistance during these studies. This investigation was supported by Research Grant GM-23152 from the National Institutes of Health, United States Public Health Service.

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