Phosphate exchange and ATP synthesis by DMSO-pretreated purified bovine mitochondrial ATP synthase

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Purified soluble bovine mitochondrial F_1F_o -ATP synthase contained 2 mol of ATP, 2 mol of ADP and 6 mol of P_i /mol. Incubation of this enzyme with 1 mM [^{32}P] P_i caused the exchange of 2 mol of P_i /mol of F_1F_o -ATP synthase. The labelled phosphates were not displaced by ATP. Transfer of F_1F_o -ATP synthase to a buffer containing 30% (v/v) DMSO and 1 mM [^{32}P] P_i resulted in the loss of bound nucleotides with the retention of 1 mol of ATP/mol of F_1F_o -ATP synthase. Six molecules of [^{32}P] P_i were incorporated by exchange with the existing bound phosphate. Removal of the DMSO by passage of the enzyme through a centrifuged column of Sephadex G-50 resulted in the exchange of one molecule of bound [^{32}P] P_i into the bound ATP. Azide did not prevent this [^{32}P] P_i ↔ ATP exchange reaction. The bound labelled ATP could be displaced from the enzyme by exogenous ATP. Addition of ADP to the DMSO-pretreated

F_1F_o -ATP synthase in the original DMSO-free buffer resulted in the formation of an additional molecule of bound ATP. It was concluded that following pretreatment with and subsequent removal of DMSO the F_1F_o -ATP synthase contained one molecule of ATP at a catalytic site which was competent to carry out a phosphate–ATP exchange reaction using enzyme-bound inorganic radiolabelled phosphate. In the presence of ADP an additional molecule of labelled ATP was formed from enzyme-bound P_i at a second catalytic site. The bound phosphate–ATP exchange reaction is not readily accommodated by current mechanisms for the ATP synthase.

Key words: adenine nucleotide, binding site, catalytic site, F_1F_o -ATPase, organic solvent.

INTRODUCTION

The proton-translocating ATP synthase is the terminal enzyme of oxidative phosphorylation in which process it synthesizes ATP from ADP and P_i . ATP synthase can be separated into two parts. The F_1 portion is the ATPase component and has the catalytic machinery for the synthesis and hydrolysis of ATP [1–4]. The F_o portion is an integral membrane protein composed of nine types of subunit in mammalian mitochondrial F_1F_o -ATP synthase [5]. F_o contains the pathway of proton translocation. The three-dimensional structures of bovine and rat liver mitochondrial F_1 and of yeast F_o have been partly determined [6–8].

F_1 is readily purified in a soluble form with high ATPase activity. It has six binding sites for adenine nucleotide, three of which have a direct role in catalysis. The remaining three sites (non-catalytic sites) may be structural or may have an indirect role in ATP hydrolysis/synthesis. Purified F_1 as isolated generally contains 3 mol of bound adenine nucleotide/mol of enzyme. Kironde and Cross [9] have proposed that two molecules of nucleotide are bound at non-catalytic sites and one molecule at a catalytic site.

Purified soluble F_1F_o -ATP synthase has lower ATPase activity than F_1 . We have shown that a purified soluble preparation of bovine mitochondrial F_1F_o -ATP synthase contained 2 mol of ATP and 2 mol of ADP/mol of enzyme [10]. Three of the four bound adenine nucleotides were exchangeable on incubation with MgATP. Bound ADP (1 mol/mol of F_1F_o) was lost readily from the enzyme on passage of the F_1F_o -ATP synthase through a centrifuged column of Sephadex G-50. It was concluded that F_1F_o -ATP synthase differed from F_1 in having all of its non-catalytic sites occupied by adenine nucleotide (2 mol of ATP and 1 mol of ADP). The ADP that was lost readily from the enzyme presumably occupied a catalytic site.

F_1 can be induced to form ATP in the presence of organic solvents such as DMSO at a single catalytic site on the enzyme [11–27]. The ATP remains firmly bound to the enzyme and is not released to the medium under these conditions. The effect of DMSO in promoting ATP synthesis by F_1 has been attributed to an increase in the affinity of the enzyme for P_i [13,20]. The decreased solvation of the P_i in DMSO would allow it to partition into a hydrophobic catalytic site where spontaneous synthesis of ATP can take place [28,29].

Because of the differences between F_1F_o -ATP synthase and F_1 in the properties of the bound adenine nucleotides, it was of interest to examine the properties of the F_1F_o -ATP synthase in the presence of DMSO to see if ATP was formed from ADP and P_i . We have found that F_1F_o -ATP synthase contained 6 mol of bound P_i /mol of enzyme and that this was completely exchangeable with exogenous P_i in the presence of 30% (v/v) DMSO. Treatment with DMSO caused loss of adenine nucleotide from the F_1F_o -ATP synthase with the retention of one molecule of ATP at a catalytic site. Removal of DMSO induced exchange of bound P_i into this remaining molecule of ATP. In contrast to F_1 , ATP synthesis did not occur with F_1F_o -ATP synthase in a solution containing 30% (v/v) DMSO. ATP was formed at a second catalytic site following transfer of the DMSO-pretreated F_1F_o -ATP synthase to its original DMSO-free buffer system.

MATERIALS AND METHODS

Materials

The sources of chemicals and enzymes have been given in [10]. Additional materials were obtained from the companies indicated: Sigma (glucose-6-phosphate dehydrogenase, Hepes and polyethyleneimine cellulose plates); Amersham ([^{32}P] P_i); Brinkman (polyethyleneimine cellulose plates); Pierce Chemical Co.

Abbreviations used: F_1 , ATPase component of the F_1F_o -ATP synthase; F_o , integral membrane component of the F_1F_o -ATP synthase.

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(bicinchoninic acid, BCA, protein assay kit); Molecular Probes (EnzChek Phosphate Assay Kit).

Preparations and assays

All preparations (F_1F_o -ATP synthase, Sephadex G-50 centrifuged columns), enzyme assays, protein determination by the Lowry method, gel electrophoresis, nucleotide binding and chase studies, luminometric measurements of bound ADP and ATP were carried out as described previously [10]. Protein was also determined by the BCA method as described by the manufacturer (Pierce Chemical Co.), since this did not give a high background reading with DMSO. Both methods were equally suitable for the assay of F_1F_o -ATP synthase protein concentrations in DMSO-free samples. Pyrophosphate was assayed by the extraction method of Tuena de Gomez-Puyou et al. [17,18]. The relative mass of the F_1F_o -ATP synthase used in our calculations was 633400 Da [10].

Phosphate assay

Assay of P_i was performed as described in the Molecular Probes EnzChek Phosphate Assay Kit. This spectrophotometric assay procedure is based on the method described by Webb [30] in which phosphate is measured using 2-amino-6-mercapto-7-methylpurine ribonucleoside and purine nucleoside phosphorylase. Quadruplicate 1 ml samples of F_1F_o -ATP synthase at 2–3 mg/ml were heated at 100 °C for 5–6 min, kept at 0 °C for 10 min, and then centrifuged in a table-top centrifuge for 10 min. The supernatants were recentrifuged at 4 °C in a microfuge for 30 min. A sample (740 μ l) was removed and assayed for P_i . Samples of F_1F_o -ATP synthase that had not been heated prior to the assay and samples carried through the assay but omitting purine nucleoside phosphorylase were used to correct any contaminating phosphate and background absorbance, respectively. These corrections amounted to less than 3% of the phosphate determined.

TLC

TLC on polyethyleneimine cellulose plates (20 $\times$ 20 cm) used 2 M LiCl, 2 M methanol and 2.5% (v/v) ethanol as solvent. Samples of ATP, ADP, AMP, phosphate and pyrophosphate were run as markers. The solvent front was allowed to reach the top of the plate (1.75–2 h) and the run was then continued for a further 2 h. The plates were air-dried overnight. Phosphate and pyrophosphate were detected by spraying with the ammonium molybdate reagent used in the assay for P_i . Nucleotide spots were located under ultraviolet light, placed in scintillation vials to which was added 1 ml of 1 M HCl. After standing for 1 h at room temperature, 10 ml of aqueous scintillant was added to the vial. The radioactivity was then determined by scintillation counting. Duplicate plates were wrapped in thin plastic film and exposed to a film (Amersham Hyperfilm-MP) or to a PhosphorImager screen (Molecular Dynamics model 524E). After an appropriate time (1–2 days) the film and/or screen was processed.

Effect of DMSO on the binding of P_i by F_1F_o -ATP synthase

F_1F_o -ATP synthase (1 mg/ml) in 50 mM Tris/acetate, pH 7.5, 0.33 M sodium acetate, 5 mM magnesium acetate, 10% (v/v) glycerol, 0.1 mM PMSF, 0.1% (w/v) benzamidine/HCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.1% (v/v) Triton RX-100 ('original buffer') was added to an equal volume of a buffer of 100 mM Hepes/KOH, pH 7.0/8 mM $MgCl_2$ /phosphate (containing [^{32}P] P_i , 10^6 – 10^7 c.p.m./ml of reaction mixture) at the indicated concentrations, with or without 60% (v/v) DMSO.

The 400 μ l reaction mixture was incubated for 15 min at 30 °C. Three 100–115 μ l aliquots were applied to previously centrifuged longer columns of Sephadex G-50 [10] equilibrated with the original buffer. The columns were centrifuged for 3.0–3.5 min. The centrifugates were then pooled and the volume made up to 350–400 μ l with the original buffer and passed through a second set of columns under the same conditions. The centrifugates were pooled and the volume made up to 350–400 μ l as before. The protein content of the pooled centrifugates was determined using 50 μ l aliquots. The content of enzyme-bound phosphate was measured by liquid scintillation counting of two 100 μ l aliquots in 10 ml of ACS (aqueous counting scintillant, Amersham). The nucleotide content of the enzyme was determined with 25–40 μ l aliquots [10]. The zero-time samples were treated similarly, but were not exposed to buffer containing DMSO and P_i .

ATP synthesis

F_1F_o -ATP synthase (1–2 mg/ml) in original buffer was added to an equal volume of a buffer to give a final concentration of 50 mM Hepes/KOH, pH 7.0, 4 mM $MgCl_2$, 1 mM P_i (containing [^{32}P] P_i , 3 – 5×10^4 c.p.m./nmol) and 30% (v/v) DMSO. The 400 μ l reaction mixture was incubated for 15 min at 30 °C. Three 115 μ l samples were removed and each applied to a previously centrifuged longer column of Sephadex G-50 equilibrated with the original DMSO-free buffer. The columns were centrifuged for 3 min. (The centrifuge tubes contained 5 μ l of 1 M sodium azide when the effect of azide on ATP synthesis was studied.) The centrifugates were pooled and made up to 400 μ l with the original DMSO-free buffer, if necessary. A second set of reaction mixtures was treated similarly. To each mixture 5 μ l of 0.1 M $MgCl_2$ was added. Nucleotide (ADP, GDP, IDP) was added at the specified concentration and the mixtures incubated at 30 °C for 10 min. The first set of reaction mixtures was treated as follows. A 50 μ l sample was used for assay of protein concentration. Two 50 μ l samples were used to determine total bound [^{32}P]phosphate plus [^{32}P]ATP. A third sample (150 μ l) was denatured by heating at 100 °C for 5 min. Denatured protein was removed by centrifugation and samples were used for the separation of radiolabelled ATP by TLC (20 μ l for each separation) and for ATP/ADP analysis by the luciferin/luciferase assay (5 μ l for each assay) [10]. For the second set of reaction mixtures, three 115 μ l samples were applied to the longer centrifuged columns of Sephadex G-50. The centrifugates were pooled, made up to 400 μ l with DMSO-free buffer, if necessary, and analysed as described above for the non-centrifuged samples.

RESULTS

P_i content of F_1F_o -ATP synthase

We previously reported that F_1F_o -ATP synthase, as prepared, contained 2 mol of ATP and 2 mol of ADP/mol. On passing the enzyme through a centrifuged column of Sephadex G-50, 1 mol of ADP/mol of F_1F_o -ATP synthase was lost [10]. The P_i content of F_1F_o -ATP synthase was measured using the sensitive nucleotide phosphorylase enzymic assay. The enzyme, as prepared, contained 6.38 ± 1.25 mol of P_i /mol of F_1F_o -ATP synthase. Two of these molecules were released by dialysis against phosphate-free buffer and 1 mol/mol of F_1F_o -ATP synthase was lost by passage through a centrifuged column of Sephadex G-50 (Table 1).

Exchange of [^{32}P] P_i from the medium with enzyme-bound P_i

F_1F_o -ATP synthase was incubated for 15 min with different concentrations of P_i containing [^{32}P]phosphate. Unbound phosphate was removed by two successive passages through centri-

Table 1 Inorganic phosphate content of F_1F_0 -ATP synthase

Phosphate was assayed as described in the Materials and methods section. The samples for assay were prepared as follows. Dialysed F_1F_0 -ATP synthase: F_1F_0 -ATP synthase (5 ml at 4 mg/ml) was dialysed at 4 °C against 1 litre of sample-preparation buffer containing 0.33 M sodium acetate for 3 h. The dialysis was continued for a further 20 h following replacement of the buffer. The dialysis membrane had a molecular-mass-exclusion limit of 8000 Da. Sephadex G-50-treated F_1F_0 -ATP synthase: the enzyme was passed through longer centrifuged columns of Sephadex G-50 [10] equilibrated with sample-preparation buffer containing 0.33 M sodium acetate. DMSO/phosphate-treated F_1F_0 -ATP synthase: F_1F_0 -ATP synthase (3 mg/ml) was incubated for 15 min with 1 mM inorganic phosphate in a buffer containing 30% (v/v) DMSO as described for ATP synthesis in the Materials and methods section except that ADP was omitted. The treated enzyme was passed successively through two columns of Sephadex G-50 before analysis. Means $\pm$ S.D. are shown.

Sample	Inorganic phosphate content (mol of P_i /mol of F_1F_0 -ATP synthase)
As prepared	6.38 ± 1.23 ($n = 8$)
Dialysed	4.16 ± 0.27 ($n = 6$)
Sephadex G-50-treated	5.17 ± 0.2 ($n = 4$)
DMSO/phosphate-treated	5.7 ± 0.2 ($n = 4$)

fuged columns of Sephadex G-50. The incubation was carried out either in DMSO-free buffer or in a buffer containing 30% (v/v) DMSO. The Sephadex G-50 was equilibrated with the

appropriate buffer. The enzyme incubated in DMSO-free buffer bound 2.06 ± 0.12 mol of labelled phosphate/mol of F_1F_0 -ATP synthase (mean $\pm$ S.D., $n = 8$; Figure 1B). Half-maximal binding was obtained at 2.5 mM phosphate. In 30% DMSO buffer, 5.99 ± 0.23 mol of labelled phosphate/mol of F_1F_0 -ATP synthase ($n = 51$) were bound (Figure 1A). Half-maximal binding occurred at 0.18–0.4 mM phosphate.

Table 1 (row 4) shows that F_1F_0 -ATP synthase treated similarly with P_i in the presence of DMSO followed by successive passage through two columns of Sephadex G-50 retained approx. 6 mol of P_i /mol of F_1F_0 -ATP synthase. Therefore, the radiolabelled phosphate of the medium in the presence of DMSO completely replaced the bound phosphate of the enzyme. In DMSO-free buffer only 2 mol of P_i /mol of F_1F_0 -ATP synthase were exchanged. Thus DMSO treatment of F_1F_0 -ATP synthase greatly increased the ability of the enzyme to exchange bound P_i with that in the medium. The [32 P]phosphate-loaded enzyme was denatured by heating and the released phosphate examined by TLC on polyethyleneimine cellulose plates. No formation of ATP or pyrophosphate was detected.

Effect of DMSO and phosphate exchange on adenine nucleotides of F_1F_0 -ATP synthase

The preparation of purified soluble F_1F_0 -ATP synthase used in these experiments after passage through a centrifuged column of

Figure 1 Binding of [32 P]phosphate (A, B) by F_1F_0 -ATP synthase and the effect on bound nucleotides (C, D) in DMSO-free (B, D) and 30% (v/v) DMSO-containing (A, C) buffer

The experiments were performed as described in the Materials and methods section.

Figure 2 Rate of release of bound phosphate following transfer of $[^{32}\text{P}]$ phosphate-loaded F_1F_0 -ATP synthase from 30% (v/v) DMSO to a DMSO-free buffer

F_1F_0 -ATP synthase was loaded with $[^{32}\text{P}]\text{P}_i$ in 30% (v/v) DMSO buffer. The enzyme was passed through a centrifuged column of Sephadex G-50 equilibrated with DMSO-free buffer. Samples were removed at timed intervals, passed through a second column of Sephadex G-50 to remove released phosphate, and then the content of enzyme-bound $[^{32}\text{P}]$ phosphate determined.

Sephadex G-50 contained 1.9 ± 0.2 mol of ATP (mean $\pm$ S.D., $n = 11$) and 1.02 ± 0.35 mol of ADP/mol of F_1F_0 -ATP synthase ($n = 11$). The enzyme lost adenine nucleotide on treatment with DMSO. This is demonstrated in Figures 1(C). Incubation of F_1F_0 -ATP synthase with phosphate in a DMSO-free system resulted in the loss of 1 mol of adenine nucleotide (0.6 mol of ATP, 0.4 mol of ADP)/mol of F_1F_0 -ATP synthase (Figure 1D). In the presence of DMSO the enzyme lost 1 mol of ATP and 1 mol of ADP/mol of F_1F_0 -ATP synthase to retain 1.05 ± 0.13 mol of ATP and 0.12 ± 0.08 mol of ADP/mol of F_1F_0 -ATP synthase ($n = 20$; Figure 1C). Thus the enzyme in DMSO, at saturating concentrations of phosphate, must contain 6 mol of P_i and 1 mol of ATP/mol of F_1F_0 -ATP synthase. The properties of the bound phosphate were examined next.

Stability of exchanged P_i

F_1F_0 -ATP synthase was loaded with $[^{32}\text{P}]$ phosphate by incubation for 15 min in the DMSO buffer system. DMSO and phosphate were then removed from the reaction mixtures by passage through a centrifuged column of Sephadex G-50 equilibrated with DMSO-free buffer. The centrifugates were pooled and incubated at 30 °C. Samples were removed at intervals and passed through a second centrifuged column to remove any $[^{32}\text{P}]$ phosphate that had been released during incubation. The remaining bound $[^{32}\text{P}]$ phosphate was determined. Passage of the enzyme through Sephadex G-50 in a DMSO-free buffer did not result in immediate loss of bound phosphate. Only 1 mol of labelled phosphate/mol of F_1F_0 -ATP synthase was lost after incubation of the phosphate-loaded enzyme for 1 h. A second molecule of phosphate was released if the incubation was continued to 2 h (Figure 2). Thus the bound phosphate of DMSO-pretreated F_1F_0 -ATP synthase is remarkably stable following transfer of the enzyme to a DMSO-free solution.

Figure 3 Effect of ADP concentration in the presence of 30% (v/v) DMSO on the bound adenine nucleotides and phosphate of DMSO-pretreated F_1F_0 -ATP synthase

F_1F_0 -ATP synthase was incubated in 30% (v/v) DMSO buffer with 1 mM $[^{32}\text{P}]$ phosphate. Unbound phosphate was then removed by passage of the solution through a centrifuged column of Sephadex G-50 equilibrated with DMSO-containing buffer. ADP was added to the column effluent and incubation continued for 30 min before the solution was passed through a second column of Sephadex G-50 equilibrated with DMSO-containing buffer. The adenine nucleotide and $[^{32}\text{P}]$ phosphate contents of the enzyme were then analysed.

Figure 4 Formation of ATP by DMSO-pretreated F_1F_0 -ATP synthase

F_1F_0 -ATP synthase was incubated with 1 mM phosphate in the presence of 30% (v/v) DMSO as described in the Materials and methods section. DMSO and unbound phosphate were removed by passage of the solution through a centrifuged column of Sephadex G-50 equilibrated with DMSO-free buffer. ADP (A, 2.5 μM ; B, 10 μM) was added to the effluent. At appropriate times after the addition of ADP the enzyme solution was heated for 5 min at 100 °C. The content of ATP was assayed by the luciferin/luciferase method.

Effect of ADP on the exchanged P_i

Since the F_1 -ATPase in 30% (v/v) DMSO-containing buffers was able to synthesize ATP from ADP and P_i [11–27], the effect of adding ADP to $[^{32}\text{P}]\text{P}_i$ -loaded F_1F_0 -ATP synthase in 30% (v/v) DMSO was examined. As shown in Figure 3, ADP caused

Table 2 Formation of ATP by DMSO-pretreated F_1F_0 -ATP synthase

F_1F_0 -ATP synthase was incubated with [32 P]phosphate in 30% (v/v) DMSO buffer or in DMSO-free buffer as described in the Materials and methods section. The solutions were passed through centrifuged columns of Sephadex G-50 and then incubated with or without ADP as indicated. The samples were heated at 100 °C prior to analysis for ATP and ADP (luciferin/luciferase assay), [32 P]P_i and [32 P]ATP (Single column) or passed through a second column of Sephadex G-50 prior to heating and assay (Two columns). See the Materials and methods section for details. All values are given as mol/mol of F_1F_0 -ATP synthase, the means $\pm$ S.D. of three separate experiments. ND, not determined. Note: total [32 P]phosphate is the sum of [32 P]P_i and [32 P]ATP.

Sample	ATP	ADP	[32 P]ATP	Total [32 P]phosphate
Single column				
Starting enzyme	1.68	2.22		
DMSO-pretreated				
0 μ M ADP	0.98 $\pm$ 0.15	0.73 $\pm$ 0.24	1.08 $\pm$ 0.28	6.22 $\pm$ 0.12
10 μ M ADP	1.93 $\pm$ 0.18	ND	1.84 $\pm$ 0.2	6.08 $\pm$ 0.15
10 μ M ADP + 3 mM azide	2.28 $\pm$ 0.11	ND	1.93 $\pm$ 0.31	6.06 $\pm$ 0.13
Not DMSO-pretreated				
0 μ M ADP	1.6 $\pm$ 0.07	1.47	0.09 $\pm$ 0.02	2.31 $\pm$ 0.22
10 μ M ADP	1.79 $\pm$ 0.05	ND	0.08 $\pm$ 0.04	2.09 $\pm$ 0.05
Two columns				
Starting enzyme	1.68	2.22		
DMSO-pretreated				
0 μ M ADP	0.96 $\pm$ 0.15	0.44 $\pm$ 0.11	0.99 $\pm$ 0.24	5.99 $\pm$ 0.26
10 μ M ADP	2.06 $\pm$ 0.14	0.94 $\pm$ 0.15	1.06 $\pm$ 0.14	3.9 $\pm$ 0.32
10 μ M ADP + 3 mM azide	1.84 $\pm$ 0.07	1.06 $\pm$ 0.17	1.17 $\pm$ 0.44	4.3 $\pm$ 0.16
Not DMSO-pretreated				
0 μ M ADP	1.64 $\pm$ 0.34	0.6 $\pm$ 0.08	0.05 $\pm$ 0.03	2.0 $\pm$ 0.05
10 μ M ADP	2.06 $\pm$ 0.04	1.17 $\pm$ 0.19	0.04 $\pm$ 0.01	2.07 $\pm$ 0.18

the release of 1 mol of P_i/mol of F_1F_0 -ATP synthase. There was no change in the levels of bound ATP or ADP. Therefore, F_1F_0 -ATP synthase did not form ATP from ADP and bound P_i in the presence of DMSO.

Synthesis of ATP from ADP and P_i by DMSO-pretreated F_1F_0 -ATP synthase

Although ATP was not formed by F_1F_0 -ATP synthase in DMSO-containing buffers (see Figure 3), removal of DMSO from the DMSO-pretreated enzyme enabled it to form ATP. This is shown in Figure 4, where the DMSO-pretreated enzyme was incubated with 2.5 or 10.0 μ M ADP following removal of DMSO and unbound phosphate by passage through a centrifuged column of Sephadex G-50. Up to 1 mol of ATP/mol of F_1F_0 -ATP synthase was rapidly formed. Concentrations of ADP up to 100 μ M did not result in the formation of more than 1 mol of ATP/mol of F_1F_0 -ATP synthase. The synthesis of ATP was investigated in more detail using radiolabelled phosphate, as shown in Table 2.

F_1F_0 -ATP synthase was loaded with [32 P]phosphate in the presence or absence of 30% (v/v) DMSO. DMSO and excess phosphate were removed by passage of the enzyme through a centrifuged column of Sephadex G-50 in DMSO-free buffer. The enzyme was then incubated with 10 μ M ADP, with and without 3 μ M sodium azide, for 10 min. The reaction was terminated by heating at 100 °C for 5 min to release bound nucleotides and phosphate (Table 2, Single column). In a second set of experiments the reaction mixture was passed through a centrifuged column of Sephadex G-50, equilibrated with DMSO-free buffer, before the heating step. This step removed any phosphate released during the incubation with ADP (Table 2, Two columns).

The enzyme used in these experiments contained 1.68 mol of ATP and 2.22 mol of ADP/mol of F_1F_0 -ATP synthase. Incubation of the enzyme with [32 P]P_i in a DMSO-free buffer resulted in the exchange of 2 mol of labelled phosphate/mol of F_1F_0 -ATP synthase (Table 2, under Not DMSO-pretreated). This labelled phosphate was not readily lost on addition of 1 mM

ATP (results not shown). Incubation with ADP gave a slight increase in the concentration of ATP but this was not significantly radiolabelled with [32 P]phosphate (Table 2, under Not DMSO-pretreated, +10 μ M ADP). Incubation of F_1F_0 -ATP synthase with labelled phosphate in DMSO buffer decreased the amount of bound ATP to 1 mol/mol of F_1F_0 -ATP synthase and 6 mol of phosphate/mol of F_1F_0 -ATP synthase were exchanged (Table 2, under DMSO-pretreated). Incubation with ADP resulted in the formation of 1 mol of ATP/mol of F_1F_0 -ATP synthase (Table 2, under DMSO-pretreated). The presence of 3 mM azide resulted in a slight increase in ATP formation. Passage of the enzyme mixture through a second centrifuged column of Sephadex G-50 after incubation with ADP reduced the level of bound [32 P]phosphate from 6 mol to about 4 mol of phosphate/mol of F_1F_0 -ATP synthase (Table 2, under Two columns, DMSO-pretreated). Thus 2 mol of phosphate/mol of F_1F_0 -ATP synthase were released by the binding of additional ADP to form ATP. The extent of labelling of the bound ATP by [32 P]phosphate found after passage through the second column of Sephadex G-50 was unexpected. Even in the absence of added ADP, the single molecule of ATP bound to the enzyme became fully labelled by [32 P]phosphate (Table 2, under Two columns, DMSO-pretreated). The additional molecule of ATP in samples which had not been passed through a second centrifuged column of Sephadex G-50 was also labelled by [32 P]phosphate, indicating *de novo* synthesis of ATP (Table 2, under Single column, DMSO-pretreated). However, this extra molecule of ATP was not labelled by [32 P]phosphate in samples which had been passed through the second column of Sephadex G-50 (Table 2, under Two columns, DMSO-pretreated). The reason for this will be discussed later.

Properties of the bound ATP in DMSO-pretreated F_1F_0 -ATP synthase

DMSO treatment removed most of the bound adenine nucleotide of F_1F_0 -ATP synthase leaving 1 mol of ATP/mol of F_1F_0 -ATP synthase. In previous work with F_1F_0 -ATP synthase it has been

Table 3 Exchangeability of bound [32 P]ATP in DMSO-pretreated F_1F_0 -ATP synthase with exogenous ATP

F_1F_0 -ATP synthase was incubated with [32 P]phosphate in 30% (v/v) DMSO buffer for 15 min and then passed through a centrifuged column of Sephadex G-50. Samples were incubated for 15 min with and without 250 μ M ATP. The reaction was terminated by heating the samples at 100 °C for 5 min. ATP and ADP were assayed by the luciferin/luciferase method, and [32 P]ATP was determined after separation by TLC (experiments under Single column). In the experiments under Two columns, the samples after incubation with and without 250 μ M ATP were passed through a second centrifuged column of Sephadex G-50 prior to heating and assay. The results are the means $\pm$ S.D. of three separate experiments and are given as mol/mol of F_1F_0 -ATP synthase. ND, not determined.

Sample	ATP	ADP	[32 P]ATP
Single column			
0 μ M ATP	1.1 $\pm$ 0.07	0.8 $\pm$ 0.34	0.9 $\pm$ 0.09
250 μ M ATP	ND	ND	0.02 $\pm$ 0.03
Two columns			
0 μ M ATP	0.99 $\pm$ 0.14	0.32 $\pm$ 0.19	0.84 $\pm$ 0.23
250 μ M ATP	1.72 $\pm$ 0.19	1.11 $\pm$ 0.21	0.02 $\pm$ 0.03

observed that all of the bound nucleotide (2 mol of ATP and 2 mol of ADP) was readily exchangeable with ATP in the medium with the exception of 1 mol of ATP/mol of F_1F_0 -ATP synthase [10]. We wished, therefore, to determine if this non-exchangeable ATP was the same as the ATP remaining after treatment with DMSO. This residual ATP became labelled with 32 P after incubation with DMSO, followed by removal of DMSO on Sephadex G-50 (Table 2). The treated F_1F_0 -ATP synthase was incubated with 250 μ M ATP. No labelled ATP was detected. All of the bound labelled ATP had been exchanged by the exogenous ATP (Table 3). The enzyme contained about 2 mol of unlabelled ATP and 1 mol of ADP/mol of F_1F_0 -ATP synthase. Thus the ATP remaining after DMSO treatment was not that which was non-exchangeable with exogenous ATP in untreated F_1F_0 -ATP synthase.

This remaining molecule of ATP in DMSO-treated F_1F_0 -ATP synthase is probably at a catalytic site since it incorporated [32 P]phosphate in a [32 P]phosphate-ATP exchange reaction. The point at which this exchange occurred was examined. F_1F_0 -ATP synthase was treated with [32 P]phosphate in DMSO in the presence and absence of 3 mM sodium azide. Part of the preparation was then heated to release bound nucleotides and phosphate. The remainder was passed before heating through a centrifuged column of Sephadex G-50 to remove DMSO and unbound adenine nucleotides and phosphate. In all cases, 1 mol of ATP was found (Table 4). However, only in those samples that had been passed through Sephadex G-50 was the bound ATP labelled by 32 P. This indicated that the [32 P]phosphate-ATP exchange reaction did not occur until DMSO had been removed

from the system. Sodium azide had no effect on the exchange reaction, although about 2 mol of P_i /mol of F_1F_0 -ATP synthase were released from the enzyme (see also Table 2).

DISCUSSION

Purified soluble F_1F_0 -ATP synthase contained about 2 mol of ADP and 2 mol of ATP/mol of enzyme [10]. The present study has shown that, in addition, 6 mol of P_i /mol of F_1F_0 -ATP synthase was present. Only two of these phosphates were exchangeable with exogenous phosphate in DMSO-free buffer solutions, whereas all six phosphates were exchangeable in the presence of 30% (v/v) DMSO buffer. The exchange of phosphate with [32 P]phosphate and the loss of adenine nucleotide from the enzyme generated a form of F_1F_0 -ATP synthase that contained 1 mol of ATP and 6 mol of [32 P]phosphate/mol of F_1F_0 -ATP synthase.

The question now arises as to the disposition of the phosphate molecules on the enzyme. It is likely that they are associated with the F_1 portion of the synthase. We have found previously that up to 6 mol of phosphate/mol of enzyme was present in the ATP-treated F_1 components from bovine mitochondria and *Escherichia coli* [23,31]. The likely sites for occupancy by phosphate are the nucleotide-binding sites, since these are designed to bind the phosphate moieties of the adenine nucleotides and, in the case of the catalytic sites, P_i itself. The F_1F_0 -ATP synthase, as prepared, has four of the six nucleotide-binding sites occupied by two molecules of ATP and two molecules of ADP. Two molecules of the phosphate could be associated with the two molecules of ADP. In their X-ray crystallography study of rat liver F_1 , Bianchet et al. [7] found that two sites on β subunits contained bound phosphate in addition to ADP. The remaining four molecules of phosphate could be bound at the two unoccupied nucleotide-binding sites. That two molecules of phosphate can occupy a single nucleotide-binding site is suggested by the work of Tuena de Gomez-Puyou et al. [17,18], who found that pyrophosphate was made by F_1 in the presence of DMSO. However, it is also possible that some phosphate is bound at other, non-nucleotide-binding, sites. It is clear that some of the molecules of bound phosphate show individual differences in behaviour. Thus in the absence of DMSO only two of the six molecules of P_i are exchangeable with exogenous phosphate. Moreover, as discussed below, some of the bound phosphate molecules can participate in a [32 P] P_i -ATP exchange reaction or phosphorylate ADP to give ATP.

As mentioned above, the F_1F_0 -ATP synthase as prepared contains 2 mol of ATP and 2 mol of ADP/mol of F_1F_0 -ATP synthase [10]. One molecule of ADP is readily lost from the enzyme, for example, on passage through a centrifuged column of Sephadex G-50. One of the remaining nucleotides is not exchangeable with exogenous ATP. In a previous study [10], we

Table 4 [32 P]Phosphate-ATP exchange occurs after removal of DMSO

F_1F_0 -ATP synthase was incubated with [32 P] P_i for 15 min in the presence or absence of 3 mM sodium azide before the reaction was terminated by heating at 100 °C for 5 min. Samples were assayed for ATP and ADP by the luciferin/luciferase method and for [32 P]ATP after TLC (Before DMSO removal). The experiment was also carried out as above except that the reaction mixtures were passed through centrifuged columns of Sephadex G-50 before heating and analysis (After DMSO removal). The results are the means $\pm$ S.D. of two separate experiments, each of triplicate values, and are given as mol/mol of F_1F_0 -ATP synthase. ND, not determined. Note: total [32 P]phosphate is the sum of [32 P] P_i and [32 P]ATP.

Sample	ATP	ADP	[32 P]ATP	Total [32 P]phosphate
Before DMSO removal	1.06 $\pm$ 0.05	2.98 $\pm$ 0.63	0	ND
Before DMSO removal + azide	1.15 $\pm$ 0.15	2.82 $\pm$ 0.28	0	ND
After DMSO removal	0.98 $\pm$ 0.11	0.18 $\pm$ 0.11	0.95 $\pm$ 0.22	5.89 $\pm$ 0.31
After DMSO removal + azide	1.16 $\pm$ 0.19	0.06 $\pm$ 0.06	0.9 $\pm$ 0.14	3.47 $\pm$ 0.25

presumed that the three nucleotides remaining after loss of the loosely bound ADP were at non-catalytic sites. The loosely bound ADP was considered to be at a catalytic site. The present data require a re-interpretation of these conclusions.

The DMSO-pretreated enzyme retained 1 mol of ATP/mol of F_1F_0 -ATP synthase. This ATP is not identical with the ATP of untreated F_1F_0 -ATP synthase, which had been observed previously not to exchange with exogenous ATP. The ATP of the DMSO-pretreated enzyme could be displaced by exogenous ATP and carried out a [32 P]phosphate-ATP exchange reaction on removal of DMSO from the system. This ATP must be at a catalytic site. Thus it is likely that the untreated ATP synthase contains 1 mol of ATP and 1 mol of ADP at non-catalytic sites, and 1 mol of ATP and 1 mol of ADP at catalytic sites. DMSO removes the nucleotides at the non-catalytic sites and the ADP at the catalytic site is lost by passage through Sephadex G-50. By contrast, treatment of F_1 containing 3 mol of adenine nucleotide/mol of F_1 with 30% (v/v) DMSO in the presence of phosphate did not result in net loss of bound nucleotide [21–23].

In contrast to F_1 [8–23], the F_1F_0 -ATP synthase did not form ATP from ADP and phosphate in the presence of DMSO. However, pretreatment with DMSO, followed by removal of this compound on centrifuged columns of Sephadex G-50 was necessary to induce net formation of ATP. The enzyme primed in this manner to form ATP contained 1 mol of ATP at a catalytic site and 6 mol of exchanged phosphate/mol. No ATP or phosphate were lost immediately on transfer to the aqueous system from the 30% (v/v) DMSO solution. However, a stoichiometric exchange of bound [32 P]phosphate into the endogenous bound ATP at a catalytic site occurred. The mechanism of this [32 P] $P_i \leftrightarrow$ ATP exchange reaction is perplexing. The bound phosphates of the synthase were not readily lost from the enzyme. Therefore, it seems unlikely that exchange involves release of phosphate from the enzyme and then stoichiometric uptake into the bound ATP. A more plausible explanation is that phosphate was bound close to the site occupied by the bound ATP such that it could undergo exchange into the ATP on removal of DMSO.

Incubation of the DMSO-pretreated synthase with ADP in a DMSO-free buffer led to the net formation of [32 P]ATP. Thus both molecules of ATP on the enzyme were radiolabelled, one by *de novo* synthesis and the other by the [32 P] $P_i \leftrightarrow$ ATP exchange reaction. However, if the enzyme was passed through a second centrifuged column of Sephadex G-50 one of the two molecules of ATP became non-radiolabelled. The only potential source of non-radioactive phosphate was that derived from the exchange of [32 P]phosphate into the bound non-radioactive ATP originally present on the enzyme. The simplest explanation for these results is that passage through the second column of Sephadex G-50 induced a reversal of the previously occurring [32 P] $P_i \leftrightarrow$ ATP exchange reaction. Thus the likely sequence of events is that exposure of the F_1F_0 -ATP synthase to DMSO induced a conformational change which caused exchange of exogenous phosphate for bound phosphate and loss of bound adenine nucleotides. Removal of the DMSO initiated [32 P] $P_i \leftrightarrow$ ATP exchange and primed the enzyme to form ATP from exogenous ADP. The enzyme was presumably in a conformation different from the initial 'resting' state. Passage through the second column of Sephadex G-50 then induced a return to the resting state and a reversal of the [32 P] $P_i \leftrightarrow$ ATP exchange reaction. The above results also imply that at least two molecules of the tightly bound phosphate present on the enzyme are at, or close to, catalytic sites.

Azide inhibits multi-site hydrolysis of ATP by F_1 . It has been proposed that this is due to the binding of inhibitory MgADP to

a catalytic site on the enzyme [32–37]. However, Harris [38] and Weber and Senior [39] have suggested that azide inhibits ATP hydrolysis of F_1 by abolishing the intersubunit co-operativity of the catalytic sites. Moreover, Weber and Senior [39] did not find bound MgADP during hydrolysis of ATP under turnover conditions in the presence of azide. Little information is available on the effect of azide on F_1F_0 -ATP synthase. However, Stiggall et al. [40] observed that ATP hydrolysis and phosphate-ATP exchange were 70% inhibited by 0.1 mM sodium azide using complex V, an F_1F_0 -ATP synthase preparation. In the present study we observed that azide did not prevent the exchange of [32 P]phosphate into the bound phosphate of the F_1F_0 -ATP synthase. Furthermore, azide had no effect on the [32 P] $P_i \leftrightarrow$ ATP exchange reaction and the *de novo* synthesis of ATP. The difference between these data and those of Stiggall et al. [40] is probably due to the absence of turnover of the system in our experiments. The exchange of [32 P]phosphate into ATP and the formation of bound ATP from bound phosphate and exogenous ADP are events probably occurring at separate sites and not involving co-operative interactions between them.

In summary, transfer of the F_1F_0 -ATP synthase to buffer containing 30% (v/v) DMSO and P_i resulted in the loss of bound adenine nucleotides from the enzyme and the formation of an enzyme species containing 1 mol of ATP and the exchange of 6 mol of P_i /mol of F_1F_0 -ATP synthase. This contrasts with the behaviour of the enzyme in DMSO-free buffer where incubation with phosphate gave a species containing 2 mol of ATP and exchange of 2 mol of P_i /mol of F_1F_0 -ATP synthase. The bound ATP of the DMSO-pretreated enzyme was relatively stable. Only 1 mol of labelled phosphate/mol of F_1F_0 -ATP synthase was lost within 60 min on transferring the enzyme to a DMSO-free buffer. The remaining ATP of the DMSO-treated F_1F_0 -ATP synthase was at a catalytic site and was readily exchangeable with exogenous ATP on removal of DMSO. Removal of DMSO led to an exchange of bound [32 P]phosphate stoichiometrically into the bound ATP. In addition, the DMSO-pretreated enzyme formed ATP from bound phosphate and added ADP at a second catalytic site when in a DMSO-free medium. ATP was not formed when the enzyme was in 30% (v/v) DMSO. This contrasts with the published behaviour of the F_1 -ATPase [11–27,31]. The exchange of exogenous phosphate into bound phosphate, the phosphate-ATP exchange reaction and the formation of ATP in a DMSO-free buffer were insensitive to azide. The bound phosphate-ATP exchange reaction catalysed by F_1F_0 -ATP synthase following pretreatment with, and then removal of, DMSO is not readily accommodated by current mechanisms for the ATP synthase.

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