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Properties of Bound Inorganic Phosphate on Bovine Mitochondrial F_1F_0-ATP Synthase[1]

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Beef-heart mitochondrial F_1F_0-ATP synthase contained six molecules of bound inorganic phosphate (P_i). This phosphate exchanged completely with exogenous $^{32}P_i$ when the enzyme was exposed to 30% (v/v) dimethyl sulfoxide (DMSO) and then returned to a DMSO-free buffer (Beharry and Bragg 2001). Only two molecules were replaced by $^{32}P_i$ when the enzyme was not pretreated with DMSO. These two molecules of $^{32}P_i$ were not displaced from the enzyme by the treatment with 1 mM ATP. Similarly, two molecules of bound $^{32}P_i$ remained on the DMSO-pretreated enzyme following addition of ATP, that is, four molecules of $^{32}P_i$ were displaced by ATP. The ATP-resistant $^{32}P_i$ was removed from the enzyme by pyrophosphate. It is proposed that these molecules of $^{32}P_i$ are bound at an unfilled adenine nucleotide-binding noncatalytic site on the enzyme. Brief exposure of the enzyme loaded with two molecules of $^{32}P_i$ to DMSO, followed by removal of the DMSO, resulted in the loss of the bound $^{32}P_i$ and in the formation of two molecules of bound ATP from exogenous ADP. A third catalytic site on the enzyme was occupied by ATP, which could undergo a $P_i \leftrightarrow$ ATP exchange reaction with bound P_i The presence of two catalytic sites containing bound P_i is consistent with the X-ray crystallographic structure of F_1 (Bianchet, *et al.*, 1998). Thus, five of the six molecules of bound P_i were accounted for. Three molecules of bound P_i were at catalytic sites and participated in ATP synthesis or $P_i \leftrightarrow$ ATP exchange. Two other molecules of bound P_i were present at a noncatalytic adenine nucleotide-binding site. The location and role of the remaining molecule of bound P_i remains to be established. We were unable to demonstrate, using chemical modification of sulfhydryl groups by iodoacetic acid, any gross difference in the conformation of F_1F_0 in DMSO-containing compared with DMSO-free buffers.

KEY WORDS: ATP synthase; adenine nucleotides; bound phosphate; phosphate exchange; ATP synthesis; mitochondria; F_1F_0 catalytic sites.

INTRODUCTION

The proton-translocating ATP synthase is the terminal enzyme of oxidative phosphorylation in which process it synthesizes ATP from ADP and inorganic phosphate. ATP synthase can be separated into two parts. The F_1 portion has the catalytic machinery for the synthesis and hydrolysis of ATP (Boyer, 1997; Hatefi, 1993; Pedersen and Amzel, 1993; Penefsky and Cross, 1991). The F_0 portion is an intrinsic membrane protein composed of nine types of subunits in mammalian mitochondrial F_1F_0-ATP synthase (Collinson *et al.*, 1994a). It contains the pathway of proton translocation. The three-dimensional structures of bovine and rat liver mitochondrial F_1 and of yeast F_0 have been partly determined (Abrahams, *et al.*, 1994; Bianchet *et al.*, 1998; Stock *et al.*, 1999).

F_1 is readily purified in a soluble form with high ATPase activity. It has six binding sites for adenine nucleotide, of which three sites have a direct role in catalysis. The remaining three sites (noncatalytic sites) may be structural or may have an indirect role in ATP hydrolysis/synthesis. Purified F_1, as isolated, generally contains 3 mol bound adenine nucleotide/mol enzyme. Kironde and

[1] Key to abbreviations: F_1, ATPase component of the F_1F_0-ATP synthase; F_0, integral membrane component of the F_1F_0-ATP synthase; DMSO, dimethyl sulfoxide; P_i, inorganic phosphate.

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Cross (1986) proposed that two molecules of nucleotide are bound at noncatalytic sites and one molecule at a catalytic site. Five to six moles of bound P_i/mole of F_1 are present on bovine mitochondrial and *E. coli* F_1 (Beharry and Bragg, 1991a, 1992a). Penefsky (1977) and Kasahara and Penefsky (1978) found a single high-affinity (K_d, 80 μM) binding site for [^{32}P]phosphate which they suggested is a catalytic site. A second nonsaturable binding site was detected. The [^{32}P]phosphate, which was bound in Penefsky and Kasahara's experiment, presumably exchanged with endogenous bound phosphate.

Purified soluble F_1F_0 has lower ATPase activity than F_1. We have shown that a purified soluble preparation of bovine mitochondrial F_1F_0 contained 2 mol ATP and 2 mol ADP/mol enzyme (Beharry and Bragg, 1996). Three of the four bound adenine nucleotides were exchangeable on incubation with MgATP. Bound ADP (1 mol/mol F_1F_0) was readily lost from the enzyme on passage of the F_1F_0 through a centrifuged column of Sephadex G-50. It was initially concluded (Beharry and Bragg, 1996) that F_1F_0 differed from F_1 in having all of its noncatalytic sites occupied by adenine nucleotide (2 mol ATP and 1 mol ADP). The ADP, which was readily lost from the enzyme, presumably occupied a catalytic site. However, our recent work (Beharry and Bragg, 2001) has shown that one of the molecules of bound ATP is present at a catalytic site in F_1F_0. Thus, F_1F_0 contains 1 molecule ATP and 1 molecule ADP at both the catalytic and the noncatalytic sites.

F_1F_0, as prepared, also contains six molecules of bound P_i (Beharry and Bragg, 2001). Incubation of F_1F_0 with $^{32}P_i$ in 30% (v/v) DMSO resulted in the exchange of all six molecules of nonradioactive P_i by $^{32}P_i$, Adenine nucleotides were also lost with the sole retention of a single molecule of ATP at a catalytic site (Beharry and Bragg, 2001). Two of the six molecules of phosphate present on the DMSO-treated enzyme were reactive following transfer of the F_1F_0 to a DMSO-free buffer. One molecule reacted with bound ATP in a $^{32}P_i \leftrightarrow$ ATP exchange reaction. A further molecule phosphorylated exogenous ADP to give bound [^{32}P]ATP (Beharry and Bragg, 2001). Thus, like F_1, F_1F_0 can be induced to form ATP by exposure to organic solvents like DMSO (Beharry and Bragg, 1991a,b, 1992a–d; Sakamoto, 1984a,b; Sakamoto and Tonomura, 1983; Cross *et al.*, 1984; Gomez-Puyou *et al.*, 1986; Kandpal *et al.*, 1987; Tuena de Gomez-Puyou *et al.*, 1993, 1995, 1998, 1999; Yoshida, 1983; Yoshida and Allison, 1986). There was a significant difference between F_1 and F_1F_0 in the effect of DMSO in inducing formation of ATP at catalytic sites. DMSO had to be present during the reaction for F_1 to make ATP. By contrast, DMSO had to be removed from the system before F_1F_0 could form ATP (Beharry and Bragg, 2001).

In contrast to the behavior of the DMSO-pretreated enzyme, incubation of F_1F_0 with $^{32}P_i$ in a DMSO-free buffer replaced only two molecules of bound P_i by $^{32}P_i$ (Beharry and Bragg, 2001), in agreement with the results of Penefsky (1977) and Kasahara and Penefsky (1978) with F_1. The F_1F_0 synthase retained 1 molecule each of ATP and ADP at noncatalytic sites, and one molecule of ATP at a catalytic site. The two molecules of $^{32}P_i$ did not undergo the $^{32}P_i \leftrightarrow$ ATP exchange reaction or form ATP with exogenous ADP (Beharry and Bragg, 2001).

In this paper, we further characterize the properties of the bound P_i of the F_1F_0-ATP synthase.

MATERIALS AND METHODS

Materials

The sources of chemicals and enzymes have been given (Beharry and Bragg, 1996). Additional materials were obtained from the companies indicated: Sigma (glucose-6-phosphate dehydrogenase; HEPES; polyethyleneimine cellulose plates); Amersham ([P^{32}]orthophosphate); Brinkman (polyethyleneimine cellulose plates); Pierce Chemical Company (BCA protein assay kit).

Preparations and Assays

The F_1F_0-ATP synthase was solubilized from bovine submitochondrial particles with deoxycholate and purified by chromatography on a column of DEAE-Sephadex CL-6B in the presence of reduced Triton X-100 and Mg^{2+}, as described (Beharry and Bragg, 1996). Azide was omitted. The specific activity was in the range of 3 to 7 μmol ATP hydrolyzed/min/mg protein. The enzyme was at least 90% pure. SDS/PAGE gels showed α, β, γ, δ, ε, a, b, c, d, e, OSCP, F_6, and A6L subunits were present, as also was the inhibitor protein. The ATPase activity of the synthase was inhibited 80% by 125 μg/ml oligomycin.

Enzyme assays, protein determination by the Lowry method, gel electrophoresis, nucleotide binding and chase studies, luminometric measurements of bound ADP and ATP, Sephadex G-50 centrifuged columns, were carried out as previously described (Beharry and Bragg, 1996). Protein was also determined by the bicinchoninic acid (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_0 protein concentrations in DMSO-free samples. The relative mass of F_1F_0-ATP synthase used in our calculations was 633,400 (Beharry and Bragg, 1996).

Thin-Layer Chromatography

Thin-layer chromatography on polyethyleneimine cellulose plates (20 cm × 20 cm) used 2 M LiCl, 2 M HCOOH, 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 then the run continued for an additional 2 h. The plates were air-dried overnight. Phosphate and pyrophosphate were detected by spraying with the ammonium molybdate reagent used in the assay of inorganic phosphate. Nucleotide spots were located under ultraviolet light, placed in scintillation vials to which was added 1 ml 1 M HCl. After standing for 1 h at room temperature, 10 ml 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 Phosphorimager). After an appropriate time (1–2 days) the film and/or screen was processed.

Binding of Inorganic Phosphate by F_1F_0

F_1F_0 (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 phenylmethylsulfonylfluoride, 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, 10^6–10^7 cpm/ml reaction mixture) at the indicated concentrations, and with or without 60% (v/v) DMSO. The 400-μl reaction mixture was incubated for 15 min at 30°C. Three 100- to 115-μl aliquots were applied to previously centrifuged longer columns of Sephadex G-50 (Beharry and Bragg, 1996) 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 μl as before. The protein content of the pooled centrifugates was determined using 50-μl aliquots. The content of enzyme-bound phosphate was measured by liquid scintillation counting of two 100-μl aliquots in 10 ml ACS. The nucleotide content of the enzyme was determined with 25- to 40-μl aliquots (Beharry and Bragg, 1996). The zero-time samples were treated similarly, but were not exposed to buffer containing DMSO and inorganic phosphate.

Release of Bound Inorganic Phosphate from F_1F_0 by ATP

The experiment was performed essentially as described above to determine inorganic phosphate and nucleotides bound to F_1F_0 in aqueous or DMSO-containing buffers. F_1F_0 was incubated for 15 min at 30°C with 1 mM [^{32}P]inorganic phosphate in the presence or absence of 30% (vl/vl) DMSO. Unbound phosphate was removed from the enzyme by passage through a centrifuged column of Sephadex G-50. ATP at the indicated concentration was added. Samples were withdrawn at timed intervals, passed through two sets of centrifuged columns of Sephadex G-50, and the bound inorganic phosphate and nucleotides analyzed as above.

Release of Bound Inorganic Phosphate from F_1F_0 by Various Agents

The experiments were carried out as described above with ATP being replaced by ADP, phosphate, or pyrophosphate, except that exogenous phosphate was removed from the system by Sephadex G-50 before the various agents were added.

Synthesis of ATP from Medium ADP and Bound P_i by DMSO Pretreated F_1F_0

F_1F_0 (0.4–0.8mg) in 400 μl original buffer with 8 mM $MgCl_2$ and 1 mM inorganic phosphate (containing 4×10^6 to 4×10^7 cpm $^{32}P_i$) was incubated for 15 min at 30°C. Three 110- to 115-μl aliquots were applied to previously centrifuged longer columns of Sephadex G-50 (Beharry and Bragg, 1996) equilibrated with the original buffer. The columns were centrifuged for 3.5 min. The centrifugates were pooled and the volume made up to 350–400 μl with the original buffer, if necessary. Sodium acetate (5 μl of 100 mM, pH 7.0) containing 100 mM Mg acetate was added followed by an equal volume of 100 mM HEPES-KOH buffer, pH 7.0, containing 60% (v/v) DMSO. The mixture was incubated at 30°C for 15 min. Three 110- to 115-μl samples were removed and passed through centrifuged columns of Sephadex G-50 as above to remove DMSO. The pooled centrifugates were made up to 350–400 μl with original buffer, if necessary. ADP (10 μM final concentration) was added to the

reaction mixtures, which were then incubated at 30°C for 15, 30, or 60 min. Each set of reaction mixtures was treated as follows. A 50-μl sample was used for assay of protein concentration. Two 50-μl samples were used to determine total bound [^{32}P]phosphate plus [^{32}P]ATP. A third sample (150 μl) was denatured by heating at 100°C for 5 min. Denatured protein was removed by centrifugation and samples were used for the separation of radiolabeled ATP by thin-layer chromatography (20 μl for each separation) and for ATP/ADP analysis by the luciferin/luciferase assay (5 μl for each assay) (Beharry and Bragg, 1996).

Carboxymethylation of Thiol Groups of F_1F_0

F_1F_0 (1.1 mg/ml) was incubated in 50 mM HEPES-KOH buffer, pH 7.0, containing 4 mM $MgCl_2$, with 100 μM iodoacetic acid containing iodo-[2-^{14}C] acetic acid (3×10^6 cpm/ml reaction mixture). DMSO (30% v/v) was present in some samples. At timed intervals, two 110-μl samples were removed from each reaction mixture and desalted by passage through longer centrifuged columns of Sephadex G-50 equilibrated with the appropriate buffer (Beharry and Bragg, 1996). The radioactivity in 25-μl samples of the eluates was determined by scintillation counting following addition of 5 ml ACS (Amersham). Protein was determined by the BCA method. The slight extent of leakage of radioisotope through the centrifuged gel matrix was corrected for by running identical samples from which F_1F_0 had been omitted.

RESULTS

Effect of Incubation with ATP or ADP on Bound Phosphate

F_1F_0 was loaded with 6 mol $^{32}P_i$/mol enzyme by incubation for 15 min with 1 mM inorganic phosphate in 30% (v/v) DMSO buffer. ATP (1 mM) was added to the solution and at timed intervals the excess phosphate and nucleotide were removed by passage through two successive centrifuged columns of Sephadex G-50 equilibrated with DMSO-containing buffer. As shown in Fig. 1A about 4 mol phosphate/mol F_1F_0 were rapidly (within 5 min) released by this treatment. The enzyme retained 2.0 ± 0.3(mean of 11 values) mol phosphate/mol F_1F_0. When F_1F_0 loaded with 2 mol $^{32}P_i$/mol enzyme in DMSO-free buffer was submitted to the same treatment, there was little immediate loss of bound phosphate. The effect of concentration of ATP on the release of phosphate in DMSO is shown in Fig. 1B. Half-maximal release of phosphate occurred at about 0.5 mM ATP. Addition of 1 mM ADP to F_1F_0 loaded with 6 mol $^{32}P_i$/mol enzyme in a buffer containing 30% (v/v) DMSO released 2 mol phosphate/mol F_1F_0 (Fig. 1).

Effect of ATP, which Can Release Bound Phosphate from F_1F_0 in DMSO, on the Adenine Nucleotide Content of the Enzyme

The bound adenine nucleotide content of F_1F_0 in the presence of DMSO was measured following addition of

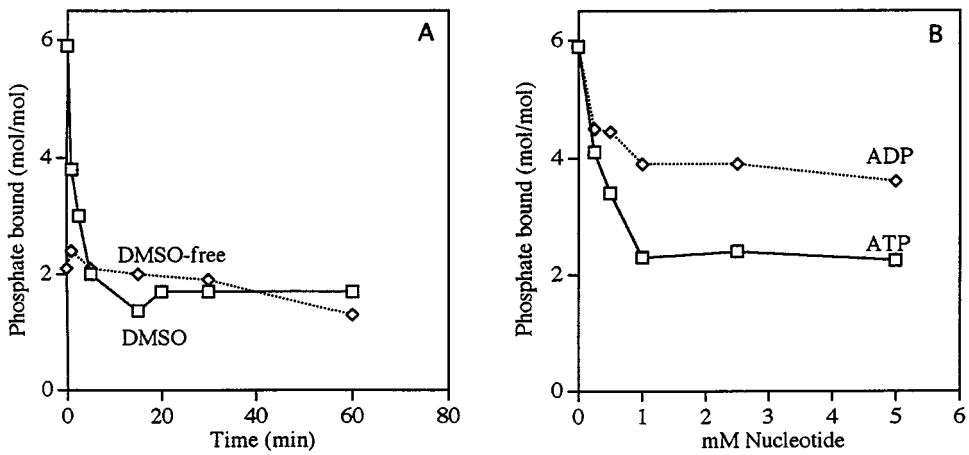

Fig. 1. Effect of ATP and ADP in releasing bound phosphate from F_1F_0. The experiments were carried out as described in section on Materials and Methods. (A) F_1 was loaded with $^{32}P_i$ in 30% (v/v) DMSO or DMSO-free buffers. ATP (1 mM) was added to the enzyme solution at zero time. (B) The experiment was carried out in 30% (v/v) DMSO as in (A). The indicated concentrations of ATP or ADP were added at zero time. The residual amount of enzyme-bound $^{32}P_i$ was determined after 30 min of incubation.

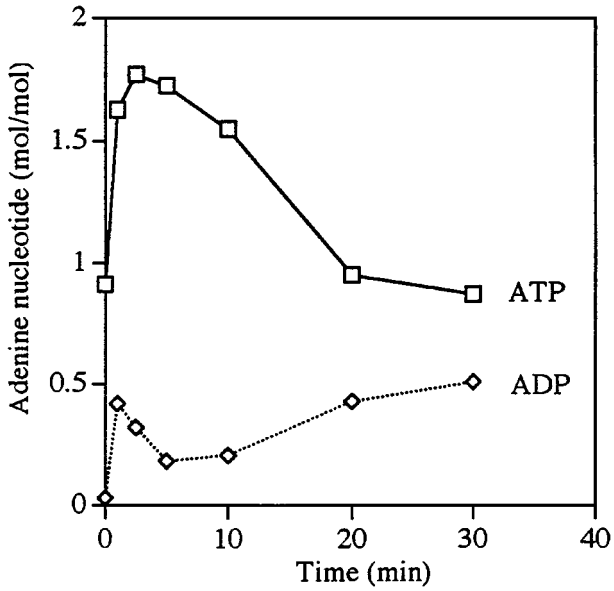

Fig. 2. Effect of addition of 1 mM ATP to phosphate-loaded F_1F_0 in 30% (v/v) DMSO on the levels of enzyme-bound ATP and ADP. The experiment was carried out as in Fig. 1A. Enzyme-bound ATP and ADP were determined as described in section on Materials and Methods.

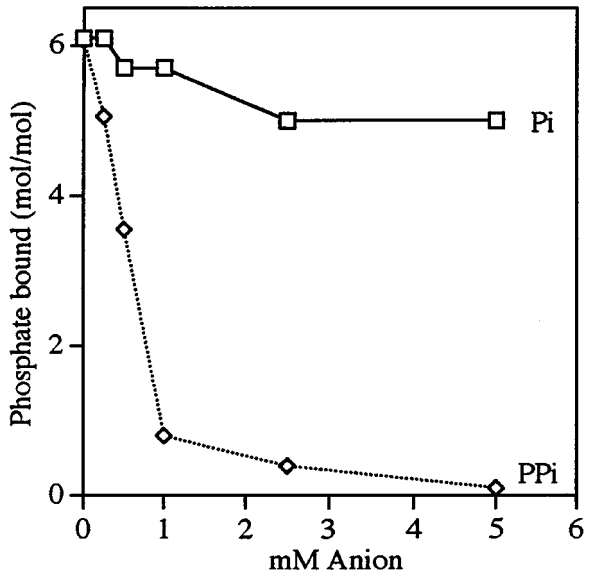

Fig. 3. Effect of inorganic phosphate (P_i) and pyrophosphate (PP_i) in releasing bound phosphate from F_1F_0 in 30% (v/v) DMSO. The experiment was carried out as described in section on Materials and Methods. F_1F_0 loaded with $^{32}P_i$ was treated at zero time with the indicated concentrations of P_i or PP_i after removal of phosphate from the medium. The residual amount of enzyme-bound $^{32}P_i$ was determined after 30 min of incubation.

ATP to release bound $^{32}P_i$, as described in the previous section. When ATP was added the release of phosphate (Fig. 1A) was accompanied by binding of almost 1 mol ATP/mol F_1F_0 followed by its subsequent hydrolysis to ADP. Thus, the ATP levels returned to initial values and about 0.5 mol ADP/mol F_1F_0 was retained by the enzyme (Fig. 2).

Effect of Phosphate and Pyrophosphate on the Release of Bound Phosphate and Adenine Nucleotides in DMSO

The ability of nonradioactive inorganic phosphate to release bound [^{32}P]phosphate from F_1F_0 in the presence of DMSO is shown in Fig. 3. Only 1 mol bound phosphate/mol F_1F_0 was exchangeable with 2.5–5.0 mM phosphate in the medium. By contrast, pyrophosphate at a concentration of 1 mM could release 87% of the bound phosphate. Phosphate at concentrations up to 5 mM had no effect on the level of bound adenine nucleotide. Treatment with 1–2.5 mM pyrophosphate removed all bound adenine nucleotide from F_1F_0 (results not shown).

Synthesis of ATP from Medium ADP and Bound P_i by DMSO Pretreated F_1F_0

F_1F_0 was loaded with 2 mol $^{32}P_i$/mol enzyme by incubation with $^{32}P_i$ in a DMSO-free buffer. Unbound $^{32}P_i$ was removed and DMSO added to give a concentration of 30% (v/v). After incubation for 15 min, the solution was freed of DMSO by passage through a column of Sephadex G-50. ADP, to give a final concentration of 10 μM, was added and incubation continued. Samples were removed for analysis of ATP formation. Table I shows that about 2 mol ATP/mol F_1F_0 were formed from bound P_i and the medium ADP. The newly synthesized ATP was not radiolabeled. Most of the bound $^{32}P_i$ was removed from the enzyme by the DMSO treatment.

Accessibility of Thiol Groups in F_1F_0 in DMSO-Free and DMSO Buffers

F_1F_0 was treated with iodo-[2-^{14}C]acetate in buffers with and without 30% (v/v) DMSO. Samples were withdrawn at timed intervals and the extent of carboxymethylation determined. Little difference in the extent of carboxymethylation between the two samples was observed (Fig. 4A). Double reciprocal plots of these data showed that 0.96 and 1.08 mol label/mol F_1F_0 was incorporated in the absence and presence of DMSO, respectively (Fig. 4B). The accessibility of the thiols of F_1F_0 to the disulfide cysteamine was determined by the method of

Table I. Formation of ATP by DMSO-Pretreated F_1F_0

Sample	mol/mol F_1F_0				
	ATP	ADP	$^{32}P_i$	[^{32}P]ATP	Total [^{32}P]phosphates
A. Starting enzyme	1.68	2.02	—	—	—
B. Not DMSO-pretreated	2.04 ± 0.36	1.02 ± 0.31	1.66 ± 0.38	0	1.66
C. DMSO-pretreated					
1. O μM ADP	1.72 ± 0.25	0.46 ± 0.09	0.31	0.08 ± 0.09	0.39
2. 10 μM ADP (15 min)	3.98 ± 0.36	ND	0.16 ± 0.06	0.03 ± 0.04	0.19
3. 10 μM ADP (30 min)	3.9 ± 0.03	ND	0.33 ± 0.04	0.02 ± 0.01	0.35
4. 10 μM ADP (60 min)	2.77 ± 0.33	ND	0.25 ± 0.04	0.01 ± 0.001	0.26

[a] F_1F_0 in its preparation buffer was incubated with [^{32}P]phosphate as described in the section on Materials and Methods. The solution, freed of phosphate by passage through a centrifuged column of Sephadex G-50, was incubated with DMSO (30% v/v) for 15 min. DMSO was then removed on a centrifuged column of Sephadex G-50. The centrifugate was incubated with and without ADP for the time specified above. The samples were heated at 100°C prior to analysis for ATP and ADP, [^{32}P]ATP, and $^{32}P_i$. (See section on Materials and Methods for details.) A control without F_1F_0 was performed to estimate any ATP in the ADP used. The results are the means of six separate experiments. ND, not determined. Note: total [^{32}P]phosphate is the sum of $^{32}P_i$ and [^{32}P]ATP.

Singh *et al.* (1993), as described in the kit purchased from Molecular Probes Inc. The thiol groups undergo an exchange reaction with this compound, liberating 2-mercaptoethylamine (cysteamine), which can be quantitated. In the absence and presence of 30% (v/v) DMSO 11.5 ± 1.2 and 12.4 ± 1.5 mol thiol groups/mol F_1F_0 (means of eight determinations), respectively, were found. The α, γ, OSCP, ε *b*, *d*, and *f* subunits of bovine mitochondrial F_1F_0 contain 6, 1, 1, 1, 1, 1, and 1 mol thiol groups/mol F_1F_0, respectively (Collinson *et al.*, 1994, 1996). Since it is unlikely that the thiol groups (1 mol/mol) of subunits c of F_0 embedded in the membrane would be accessible to the reagent, the twelve thiol groups that reacted are likely located in F_1 and the stalk linking F_1 and F_0.

DISCUSSION

Both beef-heart mitochondrial F_1 and F_1F_0 contain 6 mol bound P_i/mol F_1F_0 (Beharry and Bragg, 1991a, 2001). Previously, we showed that pretreatment of F_1F_0 with 30% (v/v) DMSO in the presence of $^{32}P_i$ resulted in the complete exchange of the bound P_i by $^{32}P_i$ (Beharry and Bragg, 2001). This observation enabled us to characterize some of the properties of the enzyme-bound phosphate. We found

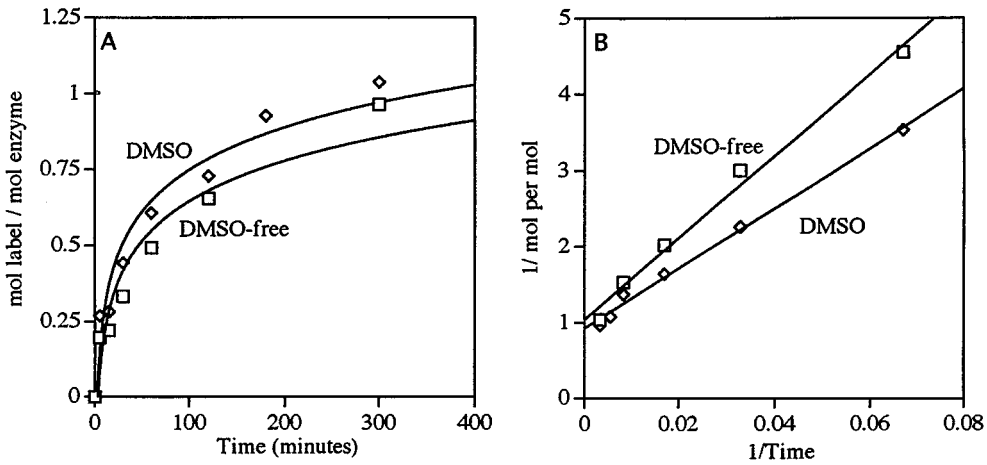

Fig. 4. Effect of DMSO on the carboxymethylation of thiol groups by [^{14}C] iodoacetate. The experiment was performed as described in section on Materials and Methods. Panel (B) shows a double reciprocal plot of the data in panel (A).

that two of the molecules of bound phosphate were associated with catalytic sites. Thus, one molecule of bound P_i formed ATP with exogenous ADP. A second molecule underwent exchange into bound ATP at a catalytic site. The present work has further characterized the properties of the enzyme-bound phosphate.

Transfer of F_1F_0 in which 2 mol $^{32}P_i$/mol F_1F_0 had been exchanged, to 30% (v/v) DMSO buffer, followed by return of the enzyme to DMSO-free buffer without phosphate, resulted in the loss of the two molecules of $^{32}P_i$. Of the remaining bound nonradioactive phosphate, two molecules were transferred to exogenous ADP to form ATP. It is likely that one of these phosphate molecules is equivalent to that which forms ATP in the DMSO-pretreated enzyme (Beharry and Bragg, 2001). The other phosphate must be at a second catalytic site, since the third catalytic site contained the ATP which undergoes exchange with bound $^{32}P_i$. This exchange reaction could not be monitored in the present experiments, since exposure to DMSO of the enzyme loaded with 2 mol $^{32}P_i$/mol F_1F_0 resulted in removal of most of the bound $^{32}P_i$. The presence of bound phosphate at two catalytic sites and of a third molecule in close association with ATP, with which it undergoes exchange, at a third catalytic site is consistent with the requirements of the binding change mechanism of ATP formation (Penefsky and Cross, 1991). The presence of two molecules of bound phosphate in catalytic sites is in agreement with the X-ray crystallographic study of rat liver F_1 by Bianchet *et al*. (1998). These workers found that two catalytic sites on β subunits of F_1 were occupied by both ADP and P_i.

The locations of the three remaining molecules of bound P_i, which are not associated with catalytic sites, have not been fully resolved. Only two mol bound P_i/mol F_1F_0 were exchangeable with exogenous $^{32}P_i$ in the absence of DMSO pretreatment. These two molecules were not displaced from the enzyme by 1 mM ATP. Similarly, two mol bound $^{32}P_i$/mol F_1F_0 remained on the DMSO-pretreated enzyme following addition of ATP. It seems likely that the ATP-nondisplaceable $^{32}P_i$ was at the same binding site in both cases. As discussed in the Introduction, a noncatalytic adenine nucleotide-binding site did not contain bound nucleotide in our preparations. Since such a site will bind ADP or ATP, it is possible that two molecules of P_i could occupy it. The ability of pyrophosphate, which binds at noncatalytic sites (Weber and Senior, 1995), to displace the ATP-resistant phosphate is consistent with this possibility. Furthermore, the results of Tuena de Gomez-Puyou *et al*. (1993, 1995), who found that pyrophosphate could be made by F_1 in DMSO, indicates that two phosphate molecules can occupy the same adenine nucleotide-binding site. The location and role of the remaining molecule of bound P_i remains to be established.

DMSO has been used as a tool in these experiments to distinguish the presence of bound phosphate involved in catalysis, *i.e*., the formation of ATP. The mechanism by which DMSO achieves this is unclear. It has been proposed that DMSO promotes ATP synthesis by increasing the affinity of the enzyme for inorganic phosphate (Sakamoto, 1984b; Kandpal *et al*., 1987). Decreased solvation of the inorganic phosphate in DMSO would allow it to partition into a hydrophobic catalytic site where spontaneous synthesis of ATP could take place (de Meis, 1989; Al-Shawi and Senior, 1992). The relevance of these suggestions in our experiments is difficult to evaluate, since the enzyme already has tightly bound phosphate. Moreover, DMSO has to be removed after the pretreatment if ATP formation is to occur. We have found previously, using sulfhydryl modification as a tool, that F_1 changes its conformation when transferred to 30% (v/v) DMSO. We were unable to demonstrate a similar change in our present experiments with F_1F_0. However, it is likely that a subtle change in conformation did occur and this could be associated with changes in conformations of the active site to favor ATP formation.

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REFERENCES

Al-Shawi, M. and Senior, A. E. (1992). *Biochemistry* **31**, 886–891.
Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994). *Nature* (London) **370**, 621–628.
Beharry, S. and Bragg, P. D. (1991a). *FEBS Lett*. **291**, 282–284.
Beharry, S. and Bragg, P. D. (1991b) *Biochem. Cell Biol*. **69**, 291–296.
Beharry, S. and Bragg, P. D. (1992a). *Biochem. J*. **286**, 603–606.
Beharry, S. and Bragg, P. D. (1992b). *Biochem. Biophys. Res. Commun*. **182**, 697–702.
Beharry, S. and Bragg, P. D. (1992c). *J. Bioenergetics Biomemb*. **24**, 507–514.
Beharry, S. and Bragg, P. D. (1992d). *Biochemistry* **31**, 11472–11476.
Beharry, S. and Bragg, P. D. (1996). *Eur. J. Biochem*. **240**, 165–172.
Beharry, S. and Bragg, P. D. (2001). *Biochem. J*. **353**, 215–222.
Bianchet, M. A., Hullihen, J., Pedersen, P., and Amzel, L. M. (1998). *Proc. Natl. Acad. Sci. USA* **95**, 11065–11070.
Boyer, P. D. (1997). *Annu. Rev. Biochem*. **66**, 717–749.
Collinson, I. R., van Raaij, M. J., Runswick, M. J., Fearnley, I. M., Skehel, J. M., Orriss, G. L., Miroux, B, and Walker, J. E. (1994a). *J. Mol. Biol*. **242**, 408–421.
Collinson, I. R., Runswick, M. J., Buchanan, S. K., Fearnley, I. M., Skehel, J. M., van Raaij, R. J., Griffiths, D. E., and Walker, J. E. (1994b). *Biochemistry* **33**, 7971–7978.

Collinson, I. R., Skehel, J. M., Fearnley, I. M., Runswick, M. J., and Walker, J. E. (1996). *Biochemistry* **35**, 12640–12646.
Cross, R. L., Cunningham, E., and Tamura, J. K. (1984). *Curr. Topics Cell Regul*. **24**, 335–344.
de Meis, L. (1989). *Biochim. Biophys. Acta* **973**, 333–349.
Gomez–Puyou, A., Tuena de Gomez Puyou, M., and de Meis, L. (1986). *Eur. J. Biochem*. **159**, 133–140.
Hatefi, Y. (1993). *Eur. J. Biochem*. **218**, 759–767.
Kandpal, R. P., Stempel, K. E., and Boyer, P. D. (1987). *Biochemistry* **26**, 1512–1517.
Kasahara, M. and Penefsky, H. S. (1978). *J. Biol. Chem*. **253**, 4180–4187.
Kironde, F. A. S. and Cross, R. L. (1986). *J. Biol. Chem*. **261**, 12544–12549.
Penefsky, H. S. (1977). *J. Biol. Chem*. **252**, 2891–2899.
Penefsky, H. S. and Cross, R. L. (1991). *Advan. Enzymology Related Areas Mol. Biol*. **64**, 173–214.
Pedersen, P. L. and Amzel, L. M. (1993). *J. Biol. Chem*. **268**, 9937–9940.
Sakamoto, J. (1984a). *J. Biochem*. **96**, 483–487.
Sakamoto, J. (1984b). *J. Biochem.* **96**, 475–481.
Sakamoto, J. and Tonomura, Y. (1983). *J. Biochem*. **93**, 1601–1614.
Singh, R., Blättler, W. A., and Collinson, A. R. (1993). *Anal. Biochem*. **213**, 49–56.
Stock, D., Leslie, A. G. W., and Walker, J. E. (1999). *Science* **286**, 1700–1705.
Tuena de Gomez-Puyou, M., Garcia, J. J., and Gomez-Puyou, A. (1993). *Biochemistry* **32**, 2213–2218.
Tuena de Gomez-Puyou, M., Sandoval, R., and Gomez-Puyou, A. (1995). *J. Biol. Chem*. **270**, 16820–16825.
Tuena de Gomez-Puyou, M., Sandoval, R., Garcia, J. J., and Gomez-Puyou, A. (1998). *Eur. J. Biochem*. **255**, 303–308.
Tuena de Gomez-Puyou, M., Perez-Hernandez, G., and Gomez-Puyou, A. (1999). *Eur. J. Biochem*. **266**, 691–696.
Weber, J. and Senior, A. E. (1995). *J. Biol. Chem*. **270**, 12653–12658.
Yoshida, M. (1983). *Biochem. Biophys. Res. Commun*. **114**, 907–912.
Yoshida, M. and Allison, W. S. (1986). *J. Biol. Chem*. **261**, 5714–5721.