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Original Article

Interaction of Beef-Heart Mitochondrial F_1-ATPase with Immobilized ATP in the Presence of Dimethylsulfoxide

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Dimethylsulfoxide [Me_2SO, 30% (v/v)] promotes the formation of ATP from ADP and phosphate catalyzed by soluble mitochondrial F_1-ATPase. The effects of this solvent on the interaction of beef-heart mitochondrial F_1 with the immobilized ATP of Agarose–hexane–ATP were studied. In the presence of Me_2SO, F_1 bound less readily to the immobilized ATP, but once bound was more difficult to elute with exogenous ATP. This suggests that not only was the binding affinity for adenine nucleotide at the first binding site affected but that adenine nucleotide binding affinity at the second and/or third sites, which interact cooperatively with the first site to release bound nucleotide, was also affected. A reduction in the binding of [^{3}H]ADP to these sites was shown. A change in the conformation of F_1 in 30% (v/v) Me_2SO was demonstrated by crosslinking and by the increased resistance of the enzyme to cold denaturation.

KEY WORDS: F_1-ATPase; adenine nucleotide binding; immobilized ATP; dimethylsulfoxide.

INTRODUCTION

In oxidative phosphorylation in mitochondria, the F_1F_0–ATP synthase complex synthesizes ATP from ADP and inorganic phosphate, coupled with proton influx across the inner membrane. F_1,[2] when separated from F_0, is a soluble enzyme complex which possesses ATPase activity, i.e., is able to hydrolyze ATP to ADP and inorganic phosphate. However, it can be induced to synthesize ATP from ADP and phosphate in the presence of 30% (v/v) Me_2SO without an energy gradient (Sakamoto and Tonomura, 1983; Yoshida, 1983; Sakamoto, 1984a,b; Cross *et al.*, 1984; Gomez-Puyou *et al.*, 1986; Kandpal *et al.*, 1987; Beharry and Bragg, 1991). It has been suggested that Me_2SO creates an environment which simulates the conditions of the mitochondrial matrix (Gomez-Puyou *et al.*, 1986). Thus, the Me_2SO–F_1 system is of considerable interest since it provides a simple system whereby some aspects of the mechanism of oxidative phosphorylation may be studied.

De Meis and coworkers (De Meis, 1984, 1989; De Meis *et al.*, 1988) have studied the potential role of solvent in affecting the solvation of phosphate and adenine nucleotide and their partitioning into a catalytic site which is suggested to undergo hydrophilic–hydrophobic transitions during catalysis. Enhanced binding of phosphate to F_1 in Me_2SO has been shown by Sakamoto (1984b) and by Kandpal *et al.* (1987). In addition, Me_2SO affects the adenine-nucleotide binding properties of mitochondrial F_1 (Beharry and Bragg, 1991).

In the present study, we have investigated the interaction of beef-heart mitochondrial F_1-ATPase with immobilized ATP in Me_2SO. It was found that in the presence of Me_2SO, F_1 binds less readily to immobilized ATP but, once bound, was more difficult to release by exogenous ATP. Decreased ADP binding at two sites on the F_1 was observed. It is proposed

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[2] Abbreviations: Me_2SO, dimethylsulfoxide; F_1, soluble ATPase from the mitochondrial ATP synthase complex; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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that this decrease in binding prevents the cooperative interaction between the sites which is responsible for the release of substrate from a catalytic site in the binding-change mechanism (Boyer 1979, 1987, 1989; Penefsky and Cross, 1991). An altered conformation of the F_1 in Me_2SO was shown by chemical crosslinking and by the increased resistance of the enzyme to cold denaturation.

MATERIALS AND METHODS

Materials

The following chemicals and enzymes were obtained from the companies indicated. Sigma: adenosine 5′-triphosphate (disodium salt), adenosine 5′-diphosphate (disodium salt), β-nicotinamide adenine dinucleotide (reduced form), L-lactate dehydrogenase (Type XI, from rabbit muscle), pyruvate kinase (Type III, from rabbit muscle; BDH Chemicals: dimethyl sulfoxide; Fisher Scientific Co.: 1,10-phenanthroline; Pharmacia P-L Biochemicals: DEAE-Sephandex G-50-120 (fine), Sephadex G-50-80, Agarose–hexane–ATP (Type 2); Amersham: [2-^{3}H]-adenosine 5′-diphosphate (ammonium salt), Aqueous Counting Scintillant, ACS; Pierce Chemical Company: 3,3′-dithiobis-(succinimidyl propionate). Aurovertin was a generous gift from Prof. R. B. Beechey.

Preparation of Beef-Heart Mitochondrial F_1-ATPase and Assays

The preparation of beef-heart mitochondrial adenosine triphosphatase, coupled assays of F_1-ATPase activity, and determination of protein concentration were carried out as described previously (Beharry *et al.*, 1990; Beharry and Gresser, 1987).

Gel Electrophoresis

SDS/PAGE was performed and proteins stained as previously described (Beharry *et al.*, 1990).

Preparation of Agarose–hexane–ATP Columns

The Agarose–hexane–ATP (AGATP Type 2, ATP linked through N6 of its adenine ring) columns were prepared and the experiments performed as described before (Beharry *et al*, 1990).

Aurovertin Fluorescence

The aurovertin fluorescence experiments were performed as described by Bragg and Hou (1987) except that the buffer was 90 mM Tris-acetate, pH 7.5, and 47 μM phosphate, with and without 30% (v/v) Me_2SO.

Binding Studies

The nucleotide-binding experiments were performed as described by Cross and Nalin (1982) and Nalin and Cross (1982). F_1 was incubated with nucleotide in a buffer of 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 47 μM phosphate, with and without 30% (v/v) Me_2SO. All experiments were performed at room temperature (22–23°C).

Crosslinking Studies

Crosslinking by 3,3′-dithiobis(succinimidyl propionate) and cupric 1,10-phenanthrolinate was carried out as described by Bragg and Hou (1980).

RESULTS

Nucleotide Binding

Beef-heart mitochondrial F_1 prepared in our laboratory contains three molecules of tightly bound adenine nucleotide per molecule of enzyme (Beharry and Bragg, 1991). One of these molecules can be readily removed by high concentrations of GTP and is therefore classed as an exchangeable, and presumably, catalytic site (Kironde and Cross, 1986, 1987). The other two nonexchangeable molecules of adenine nucleotide are at noncatalytic sites. In addition to the sites containing bound nucleotide, there are three sites which can be filled by the nonhydrolyzable adenine nucleotide analog adenylyl-5′-imidodiphosphate. The affinity of F_1 for the analog was lowered at two of the sites when the enzyme was in 30% (v/v) Me_2SO (Beharry and Bragg, 1991).

The reduced affinity shown by F_1 for the adenine nucleotide analog was confirmed using ADP, the natural substrate of the synthesis reaction (Fig. 1). Approximately 4 mol [^{3}H]ADP/F_1 and 2 mol [^{3}H]ADP/F_1 were bound in the absence and presence of 30% (v/v) Me_2SO, respectively. Presumably, in the aqueous system, the [^{3}H]ADP filled the three vacant nucleotide binding sites and displaced the bound nucleotide from the exchangeable site. In Me_2SO, there is a reduced affinity for ADP at two of the binding sites.

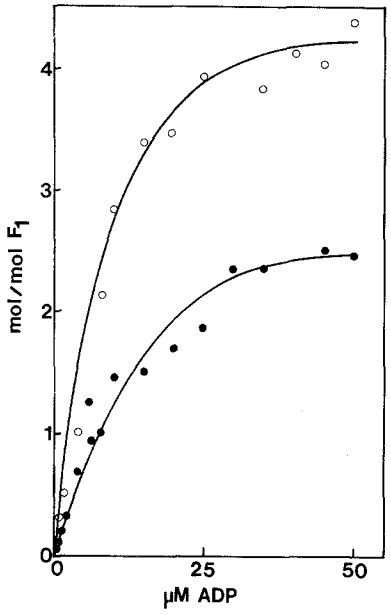

Fig. 1 Binding of [^{3}H]ADP to F_1 in the absence (○) and presence (●) of 30% (v/v) Me_2SO. F_1 (2.5 μM upper curve, 2.3 μM lower curve); was incubated with ADP for 60 min in a buffer with and without Me_2SO. Samples were freed of unbound ADP using centrifuged columns of Sephadex G-50 equilibrated with and without Me_2SO. Bound nucleotides were determined as described in Materials and Methods. The extent of binding expressed as mol ADP bound per mol F_1 is plotted versus the concentration of ADP in the incubation medium.

Binding of F_1 to Agarose–hexane–ATP

We have recently shown (Beharry *et al.*, 1990) that beef-heart mitochondrial F_1 will bind to the immobilized ATP moiety of Agarose–hexane–ATP columns, and that the bound F_1 can be eluted with ATP. The immobilized ATP is hydrolyzed to ADP and phosphate, and elution of F_1 probably involves cooperative interactions between catalytic sites on the enzyme. The interaction of F_1 with columns of Agarose–hexane–ATP was investigated in the presence and absence of 30% (v/v) Me_2SO (Fig. 2 and Table I). The binding and elution profiles of F_1 were similar in both systems. Less F_1 was bound in Me_2SO buffer than in the completely aqueous buffer (Table I) (NB: enzyme activity and protein concentration, as shown by SDS-polyacrylamide gel electrophoresis, closely paralleled one another). This is in agreement with the reduced affinity for adenine nucleotides shown by F_1 in Me_2SO buffers (Beharry and Bragg, 1991) (Fig. 1). However, only 42% of the bound activity was eluted by ATP in the Me_2SO system compared with 71% when the organic solvent was absent (Table I). The reduced ability of ATP to release bound F_1 may be due to the decreased ability of bound F_1 to bind a second and/or third ATP molecule in the Me_2SO system. Alternatively, the cooperativity between adenine nucleotide-binding sites may be affected by the presence of Me_2SO.

Consistent with these interpretations were the results from a second type of experiment. F_1 was allowed to bind to immobilized ATP in aqueous buffer. The columns were then equilibrated with buffer, with and without 30% (v/v) Me_2SO, and eluted with ATP in the corresponding buffer (Fig. 3). Phosphate was not present in the buffers. As before, half as much activity was eluted with the ATP/Me_2SO buffer as compared with the ATP/aqueous buffer. It is thus apparent that whether F_1 is exposed to Me_2SO before or after binding to immobilized ATP, there is a reduced affinity for incoming ATP and/or a decrease in cooperativity between binding sites. In an attempt to distinguish between these alternatives, the fluorescence behavior of F_1 with aurovertin D was examined.

Aurovertin Fluorescence

Aurovertin D binds to F_1 to form a complex the fluorescence of which is affected by adenine nucleotides. Vazquez-Laslop *et al.* (1989) have shown that the fluorescence behavior of the aurovertin D·F_1 complex on addition of ATP reflects the hydrolysis of the ATP at a single catalytic site. In the presence of aurovertin D cooperative mechanisms are blocked (Lunardi *et al.*, 1986).

Addition of aurovertin D to F_1 in buffer resulted in an increase in fluorescence intensity as the aurovertin D·F_1 complex was formed (Fig. 4). The increase was less in the Me_2SO medium. $MgCl_2$ (0.25 mM) caused partial quenching of fluorescence. An increase in fluorescence intensity was then produced by the addition of 1.25 μM ATP. The rate at which the fluorescence increased was markedly slower in the Me_2SO system than in the entirely aqueous system. The addition of a high level (2 mM) of ATP resulted in the quenching of fluorescence, as has been observed by others (Pougeois *et al.*, 1979). These experiments show that ATP-induced effects occurring at a single catalytic site are influenced by the presence of Me_2SO. Therefore, the results described in the previous sections are consistent with there being a major effect of Me_2SO on the binding of adenine nucleotide.

Crosslinking

The Me_2SO-mediated changes should be reflected

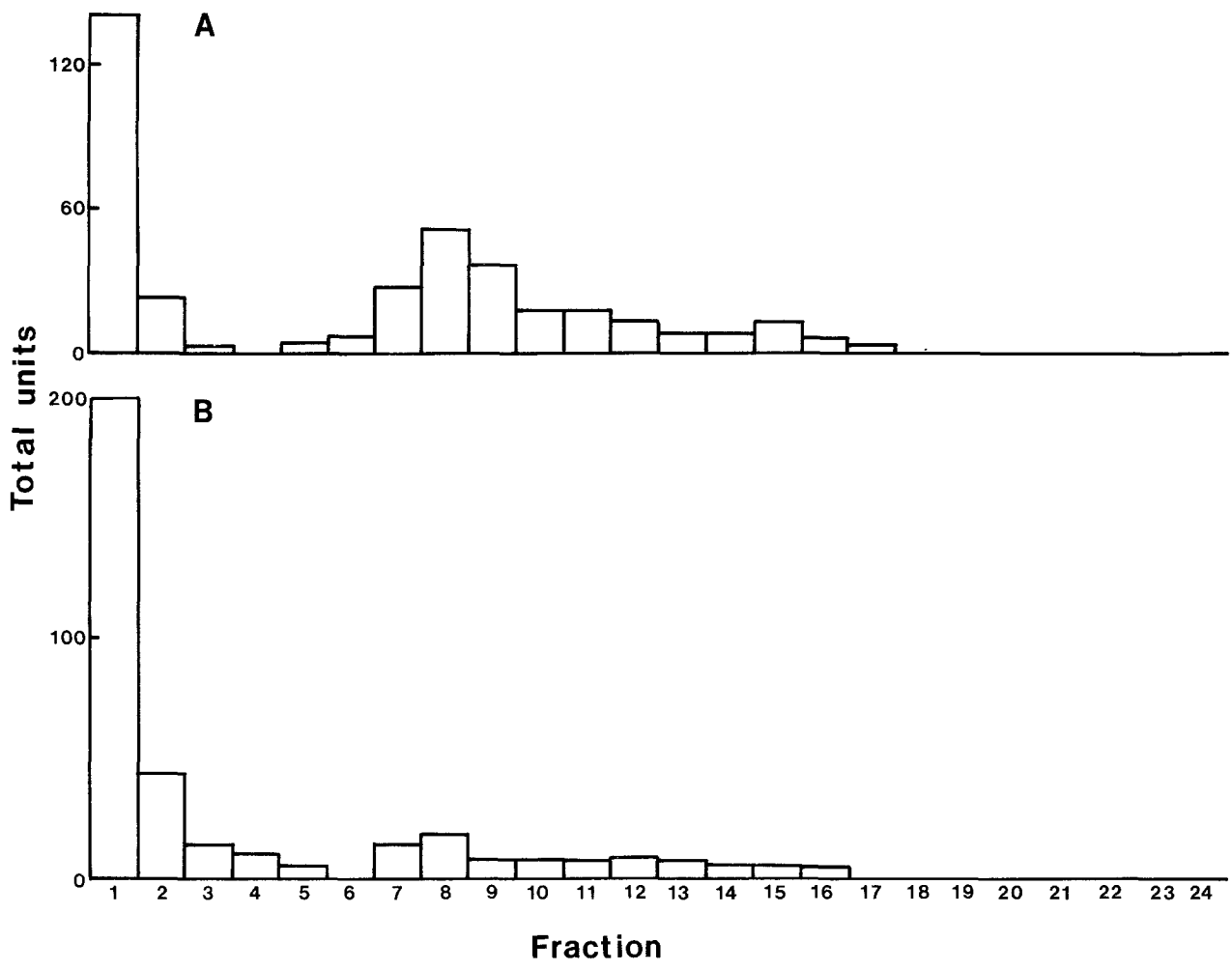

Fig. 2. Elution by ATP of F_1 ATPase from Agarose–hexane–ATP. A 1.0-ml portion of F_1, 0.75 mg of protein, containing 500 units of activity, was applied to a 1-ml Agarose–hexane–ATP (Type 2) column. The fractions were eluted as follows (see Materials and Methods): 1–5, buffer washes, with centrifugation; 6, buffer containing 1.0 mM AMP, with centrifugation; 7–14, buffer containing 10 mM ATP, without centrifugation; 15–16, as for 7–14, but with centrifugation; 17–20, buffer containing 2 M urea, without centrifugation, and 21–24, buffer containing 2 M guanidine HCl, without centrifugation. The buffer used was 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 47 μM phosphate in the absence (A) and presence (B) of 30% (v/v) Me_2SO. F_1 was applied and eluted in the same buffer except where the mentioned additions were made. A unit of activity is the amount of enzyme causing a change in the absorbance at 340 nm of 1/min in the coupled assay.

in an altered conformation of the enzyme. This was verified by crosslinking studies.

Treatment of the enzyme with cupric 1,10-phenanthrolinate in an entirely aqueous medium generated several crosslinked products, of which the two most prominent are designated 1 and 2 in Fig. 5A. The disulfide linkages formed by the crosslinker were cleaved by 2-mercaptoethanol, and the products of cleavage were recognized by sodium dodecyl sulfate gel electrophoresis in the second dimension. Product 1 yielded α and γ subunits; product 2 gave the γ subunit only. Higher molecular weight products containing α and/or γ subunits were also present (Fig. 5A). By contrast, little crosslinking was observed when the reaction was performed in 30% (v/v) Me_2SO (Fig. 5B). Presumably, the alignment between sulfhydryl groups on the α and γ subunits, which enables a disulfide crosslinkage to be formed in aqueous medium, is altered in 30% (v/v) Me_2SO. To determine if the lack of crosslinking of F_1 by cupric 1,10-phenanthrolinate in Me_2SO was due to the inability of the reagent to oxidize sulfhydryl groups in the organic solvent, we examined the effect of cupric 1,10-phenanthrolinate pretreatment on the reaction of cysteine with DTNB

Table I. Elution of F_1 from agarose–Hexane–ATP (Type 2) in Buffer with and Without 30% (v/v) Me_2SO[a]

	Aqueous buffer	30% (v/v) Me_2SO buffer
1. Total activity applied	500 units	500 units
2. Activity not bound, Fractions 1–5	230 units	305 units
Activity bound	270 units	195 units
3. Activity eluted with ATP buffer	190 units	82 units
% Recovery	71%	42%

[a]Data taken from Fig. 2.

(Beharry and Bragg, 1989). The cupric 1,10-phenanthrolinate caused rapid loss of reaction of cysteine with DTNB, both in the presence and absence of 30% (v/v) Me_2SO, due to the oxidation of the sulfhydryl group of cysteine. Thus, the lack of crosslinking of F_1 in Me_2SO is unlikely to be due to the inability of the reagent to oxidize the sulfhydryl groups to form the crosslink. The amino-reactive crosslinking agent 3,3′-dithiobis(succinimidyl propionate), which requires less critical alignment between groups for crosslinking, gave a large number of different crosslinked products of the subunits of F_1 (results not shown). There was no was no difference between entirely aqueous and 30% (v/v) Me_2SO media in the nature and amount of

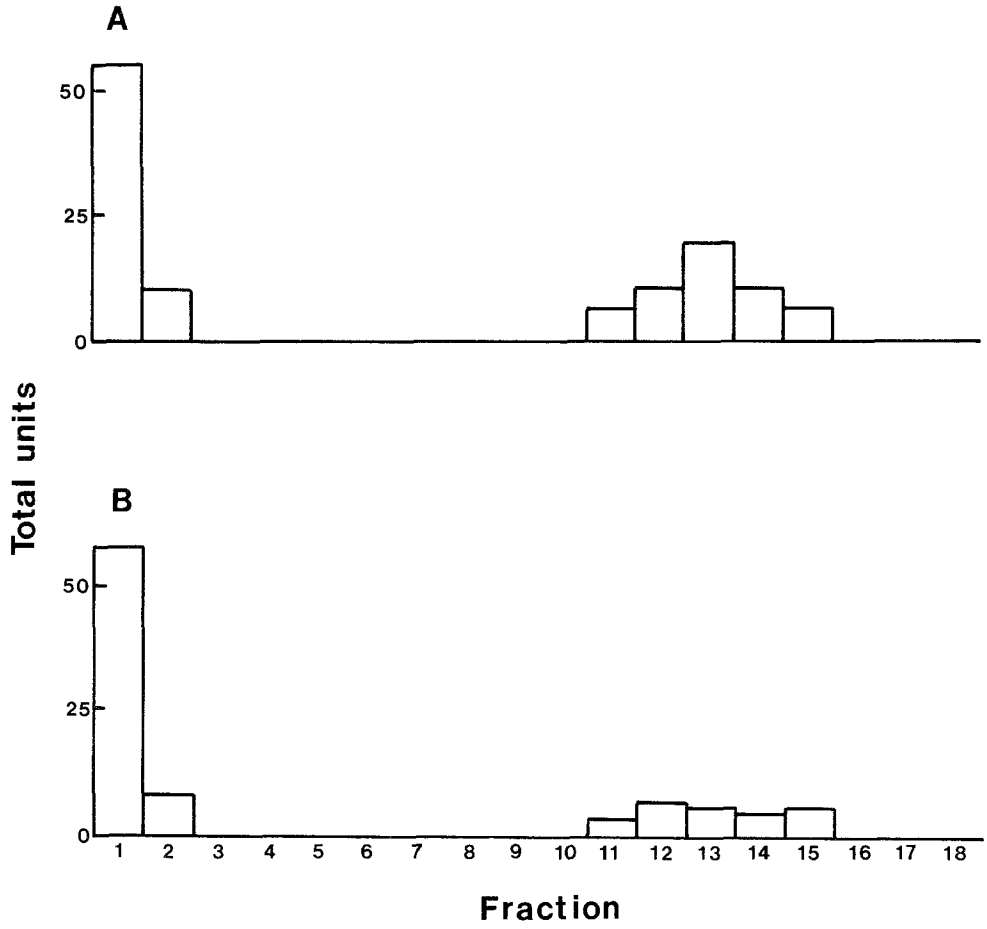

Fig. 3. Elution by ATP and F_1 ATPase from Agarose–hexane–ATP after Me_2SO washes. A 1.0-ml portion of F_1, 0.71 mg of protein, containing 200 units of activity, was applied to a 1-ml Agarose–hexane–ATP (Type 2) column. The fractions were eluted as follows (see Materials and Methods): 1–5, buffer washes; 6–9, buffer without and with 30% (v/v) $MeSO_4$; 10, buffer with 1 mM AMP ± 30% (v/v) Me_2SO; 11–14, buffer with 10 mM ATP ± 30% (v/v) Me_2SO; 15–18, buffer with 2 M guanidine hydrochloride. The buffer used was 90 mM Tris-acetate, pH 7.5, and 1.6 mM $MgSO_4$ with (B) and without (A) 30% Me_2SO (v/v) as indicated. A unit of activity is the amount of enzyme causing a change in absorbance at 340 nm of 1/min in the coupled assay.

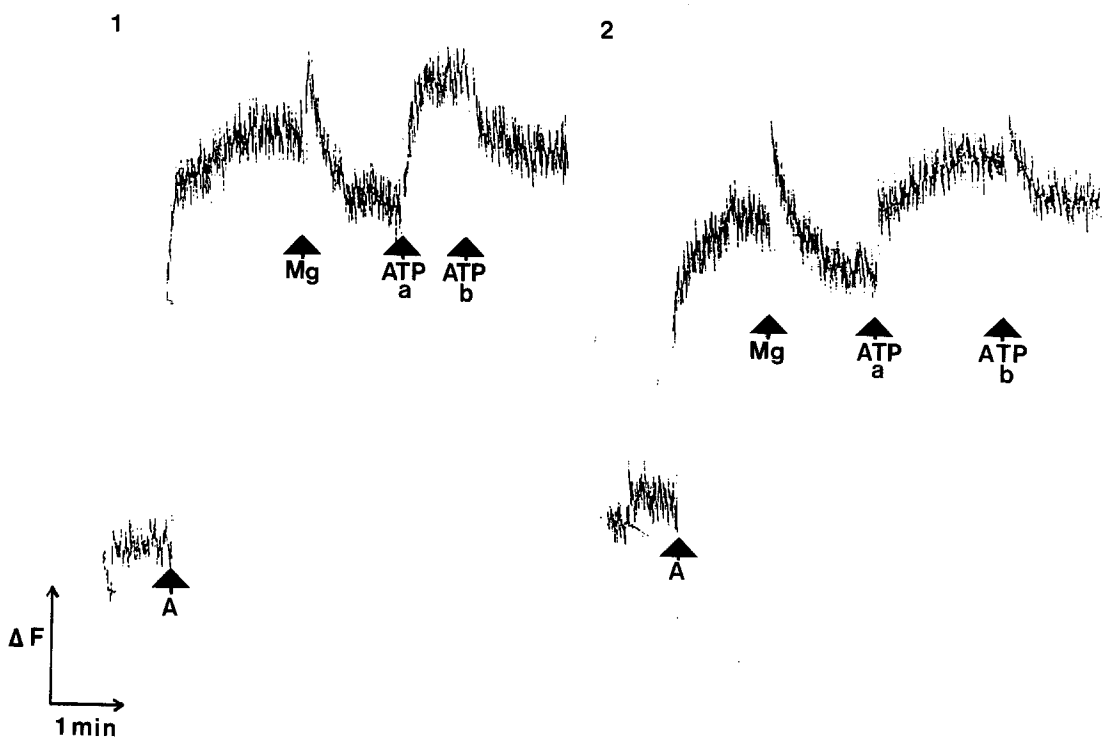

Fig. 4 Effect of ATP and Me_2SO on the fluorescence intensity of the aurovertin D-F_1 ATPase complex. Fluorescence measurements were made as described in Materials and Methods using 0.1 mg F_1 protein/ml buffer with (2) and without (1) 30% (v/v) Me_2SO. The volume of buffer was 2 ml. A, 3 μl aurovertin D at 1.25 mM; Mg, 0.5 μmol $MgSO_4$; ATP a, 25 nmol ATP; ATP b, 4 μmol ATP added.

crosslinked products formed by this latter crosslinker, thus indicating that in contrast to cupric 1,10-phenanthrolinate, it was not able to detect the subtle changes in conformation induced by Me_2SO which were evidenced by altered nucleotide-binding properties.

Effect of Me_2SO on the Stability of F_1

F_1, once detached from F_0, is a very cold-labile enzyme, as seen in Fig. 6 and in early studies of the enzyme (Pullman *et al.*, 1960). Cold denaturation was completely prevented by the presence of 30% (v/v) Me_2SO. The enzyme showed no loss of activity over the time course of the experiment. This suggests that in the presence of Me_2SO, the F_1 may be in a form similar to that in the cold-stable F_1F_0 complex.

DISCUSSION

The experiments described in this paper confirm and extend our previous proposals (Beharry and Bragg, 1989, 1991) that the presence of Me_2SO affects the conformation of beef-heart mitochondrial F_1 and the nucleotide-binding properties of the enzyme.

The altered conformation of the enzyme was shown by an increased resistance to cold denaturation and by an alteration in the ability to crosslink subunits with cupric 1,10-phenanthrolinate. This agent induced the formation of a crosslink between one of the two sulfhydryl groups on an α subunit (cysteine-201 or cysteine-251) and the sulfhydryl group of cysteine-78 of the γ subunit. The conformational change induced by Me_2SO resulted in the loss of the alignment between the two cysteine residues which permitted crosslinking. These results are consistent with our previous study of sulfhydryl modification of F_1 which showed that sulfhydryl groups on the α, γ, and ε (cysteine-18) subunits were in close proximity such that only one could be modified by 5,5′-dithiobis(2-nitrobenzoic acid) in the native enzyme (Beharry and Bragg, 1989). However, 30% Me_2SO (in the presence of ATP) resulted in a conformational change so that the cysteine residues on the α and ε subunits could be simultaneously modified by this reagent. These two sets of data lead to a model in which one of the three α subunits and the γ and ε subunits interface in the region of their cysteine residues, and it is this region which relaxes in the presence of 30% (v/v) Me_2SO.

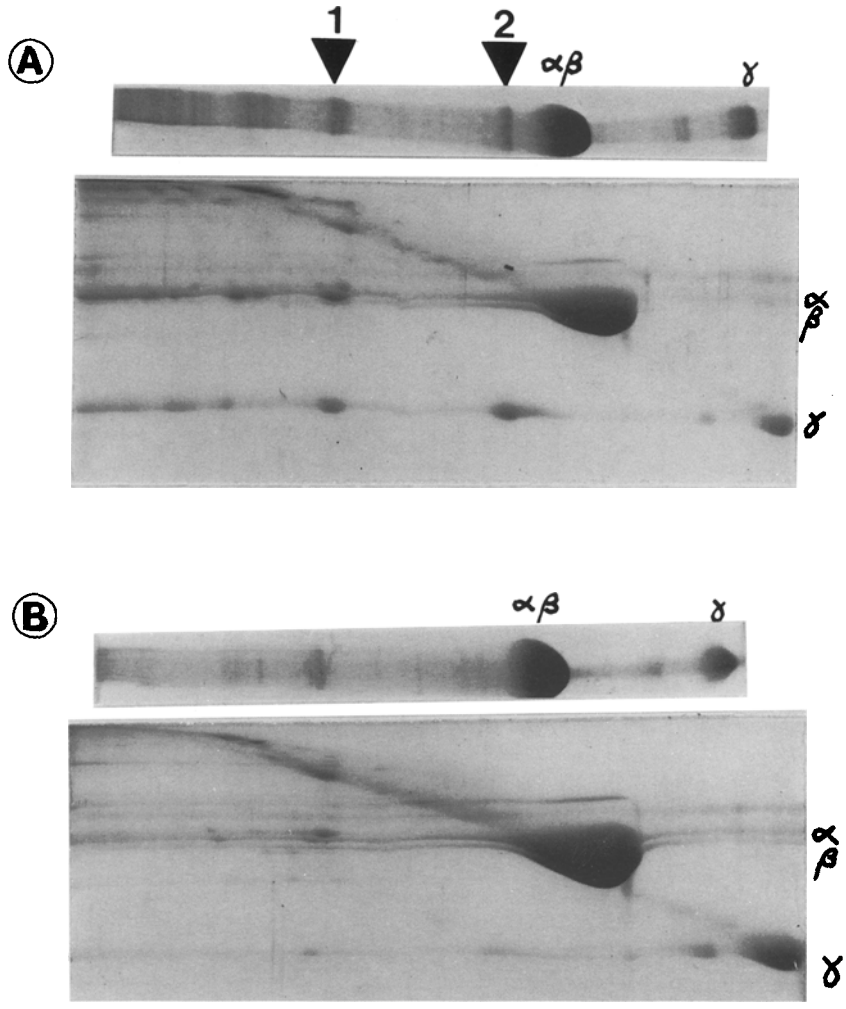

Fig. 5 Crosslinking of F_1 by cupric, 1,10-phenanthrolinate in the absence (A) and presence (B) of 30% (v/v) Me_2SO. Crosslinking was carried out as described by Bragg and Hou (1980), and the products resolved by two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. The direction of migration is from left to right in the first dimension and from top to bottom in the second dimension. A Coomassie blue-stained duplicate strip of the first-dimension gel is placed above the two-dimensional gel for identification of crosslinked products. The subunits arising from the cleavage of these products with 2-mercaptoethanol are seen in the two-dimensional gel directly below the labelled bands. The positions of migration of the subunits of F_1 are indicated.

The alteration in the conformation of F_1 is associated with, but not necessarily the cause of, a change in the nucleotide-binding properties of F_1. Less [^{3}H]ADP binds to F_1 in 30% (v/v) Me_2SO. The decrease from 4 mol/mol F_1 to 2 mol/mol F_1 in the amount of labeled ADP which can bind suggests that two potential adenine nucleotide-binding sites must be unoccupied in the presence of Me_2SO. A reduction in the binding affinity at these two sites could account for the fact that ATP formed by F_1 in Me_2SO is not released from the enzyme even in the presence of excess ADP and phosphate (Sakamoto and Tonomura, 1983), and for the greater difficulty of releasing F_1 bound to a column of Agarose–hexane–ATP in the presence of Me_2SO.

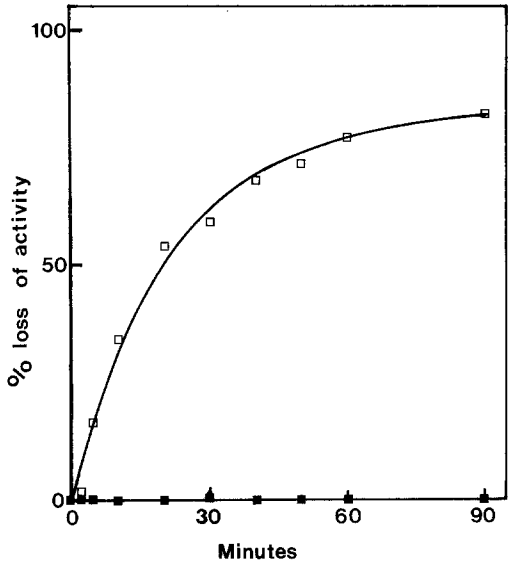

Fig. 6 Cold denaturation of F_1 in the presence (●) and absence (○) of 30% (v/v) Me_2SO. The enzyme (0.94 mg protein/ml) was incubated at 0°C. At intervals, samples were removed for assay of ATPase activity. The initial activities without and with Me_2SO were 96 and 48 μmol ATP hydrolyzed/min/mg protein.

Both situations require the release of the ATP by cooperative interactions involving the binding of adenine nucleotide at a second and/or third site (Boyer, 1979, 1987, 1989; Penefsky and Cross, 1991). A reduction of binding affinity for adenine nucleotide at the second or third site, as shown above, would affect the release mechanism.

That the binding affinity for ATP at a catalytic site is affected is supported by our results with the aurovertin D·F_1 complex. The presence of Me_2SO decreases the rate of increase of fluorescence seen on addition of ATP. The work of Vazquez-Laslop *et al.* (1989) and of Lunardi *et al.* (1986) suggests that the fluorescence increase is due to the hydrolysis of ATP at a *single* catalytic site and is independent of cooperative interactions. A reduced binding affinity for ATP in Me_2SO could account for our data.

CONCLUSION

The results presented here are consistent with a reduction in adenine nucleotide binding affinity at all exchangeable sites on F_1. The decrease in adenine nucleotide binding at the sites required to release product from a catalytic site could account for the stronger retention of F_1 on columns of Agarose–hexane–ATP and for the lack of release of product ATP formed by F_1 from ADP and phosphate in Me_2SO. These data do not exclude the possibility that the altered conformation of F_1 in 30% (v/v) Me_2SO may also be unfavorable for cooperative interactions between adenine nucleotide binding sites.

ACKNOWLEDGMENTS

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