Changes in the adenine nucleotide and inorganic phosphate content of *Escherichia coli* F₁-ATPase during ATP synthesis in dimethyl sulphoxide

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Escherichia coli F₁-ATPase contained 2.9 ± 0.1 mol of adenine nucleotide and 3.1 ± 0.3 mol of P_i/mol of enzyme. After preincubation with ATP, the nucleotide and phosphate contents were 5.6 and 6.0 ± 0.5 mol/mol of enzyme respectively. The F₁-ATPase was induced to synthesize ATP in the presence of 30% (v/v) dimethyl sulphoxide (Me₂SO). The ATP originated from endogenous bound ADP. The bound adenine nucleotide and P_i contents of the enzyme during the time course of ATP synthesis were investigated by using F₁-ATPase which had been preincubated with ATP. We show that the process of ATP synthesis in Me₂SO involves (i) an initial rapid loss of nucleotide from the enzyme, the process being facilitated by exogenous P_i, (ii) a rapid loss of P_i from the enzyme, at least in the absence of exogenous P_i, (iii) re-binding of a portion of the lost nucleotide, and (iv) synthesis of ATP from bound ADP and exogenous P_i. It is proposed that transfer of the F₁-ATPase to the Me₂SO medium induces a change in the conformation of the enzyme to a form favouring ATP synthesis.

INTRODUCTION

Escherichia coli F₁F₀, the proton-translocating ATP synthase, is the terminal enzyme of oxidative phosphorylation, in which process it synthesizes ATP from ADP and P_i. F₁ may be separated from F₀ as a soluble enzyme able to hydrolyse ATP to ADP and P_i. It consists of five different subunits (α – ϵ) in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Senior, 1988; Futai *et al.*, 1989; Penefsky & Cross, 1991). F₁, when isolated, usually contains 3 mol of adenine nucleotide/mol of F₁ (Maeda *et al.*, 1976; Bragg & Hou, 1977). Three other nucleotide-binding sites are present, which may be filled with nucleotide by incubating F₁ with adenosine 5'-[β -imidotriphosphate (Wise *et al.*, 1984), ATP (Bragg *et al.*, 1982; Bragg, 1984; Issartel *et al.*, 1986) or with ADP in the presence of 40 mM-Mg²⁺ (Lunardi *et al.*, 1981). Incubation of the *E. coli* F₁ with ADP at 2.5–5 mM-Mg²⁺ fills two sites only (Lunardi *et al.*, 1981; Wise *et al.*, 1981). A single site is filled by ADP in the absence of bivalent cation (Bragg & Hou, 1977; Lunardi *et al.*, 1981; Wise *et al.*, 1981; Bragg, 1984). Perlin *et al.* (1984) showed that, of the six nucleotide-binding sites, three were specific for adenine nucleotides, whereas the remaining three sites could bind guanine or inosine nucleotides as well. The latter sites were catalytic sites, likely to be located on the β subunits. The non-catalytic sites, specific for adenine nucleotides, are on the α subunits (Dunn & Futai, 1980; Perlin *et al.*, 1984). Kironde & Cross (1986, 1987), with mitochondrial F₁, have shown that the isolated enzyme contains 2 mol of adenine nucleotide bound at non-catalytic sites and 1 mol of adenine nucleotide at a catalytic site. They introduced the symbol F₁[2,1] to describe this state of site occupancy. The site occupancy of the *E. coli* F₁ has not been defined.

Mitochondrial F₁ can be induced to synthesize ATP from ADP and P_i in the presence of 30% (v/v) dimethyl sulphoxide (Me₂SO) (Sakamoto & Tonomura, 1983; Yoshida, 1983; Sakamoto, 1984a,b; Gomez-Puyou *et al.*, 1986; Kandpal *et al.*, 1987; Beharry & Bragg, 1991a,b). The ATP so formed is not released from the enzyme. Sakamoto (1984b) and Kandpal *et al.* (1987) have suggested that ATP synthesis is favoured primarily by Me₂SO promoting the binding of P_i to F₁, rather than by

promoting the formation of bound ATP from bound ADP and phosphate. We have shown recently (Beharry & Bragg, 1991a) that Me₂SO also affects the adenine-nucleotide-binding properties of mitochondrial F₁ with a decrease in the affinity for ADP at one of the catalytic sites. Exogenous ADP can still be bound by a non-catalytic site. The ATP synthesized in Me₂SO is formed from the endogenous ADP at the catalytic site of the F₁[2,1] enzyme.

E. coli F₁ has proved useful for many studies of the mechanism of the ATP synthase (Senior, 1988; Futai *et al.*, 1989; Penefsky & Cross, 1991). Formation of ATP by *E. coli* F₁ in Me₂SO has not been shown. In the present paper, we show that *E. coli* F₁ contains bound P_i and that ATP synthesis from endogenous bound ADP and P_i in the medium is induced in the presence of Me₂SO. This process involves (i) an initial rapid loss of bound nucleotides and of endogenous P_i, (ii) re-binding of a portion of the lost nucleotide, but not of P_i, and (iii) synthesis of ATP from bound ADP and P_i in the medium.

MATERIALS AND METHODS

Preparation of F₁-ATPase

The F₁-ATPase of *E. coli* ML308-225 was prepared as described previously (Bragg & Hou, 1986), except that the fractions from the aminohexyl-Sepharose 4B column were applied to a 15–25% (w/v) sucrose gradient prepared in a buffer consisting of 50 mM-Tris/HCl, 2 mM-EDTA and 4 mM-ATP, pH 7.5. Gradient fractions containing F₁ were pooled, and an equal volume of saturated (NH₄)₂SO₄ solution, pH 8, was added to precipitate the enzyme. The suspension containing the precipitated enzyme was stored at 4 °C. The enzyme was obtained, as needed, by sedimentation (10000 g for 10 min). It was dissolved in 100 mM-Tris/acetate, pH 6.8, and desalted by passage through a Sephadex G-50-80 centrifuged column equilibrated with the same buffer (Penefsky, 1977). The *M_r* of F₁ used in calculations was 381 000 (Senior, 1988).

Assays

ATPase activity was measured at 37 °C in the presence of

2.5 mM-MgCl₂ as described previously (Bragg & Hou, 1975). The coupled assay of ATPase activity and the determination of protein concentration have been described (Beharry & Gresser, 1987). Analysis of P_i was by the procedure of Lanzetta *et al.* (1979), using Malachite Green (note: Sterox was replaced by Brij 35, with satisfactory results). For analysis, 0.55 ml at 2 mg of protein/ml or 1.1 ml of F₁ at 1 mg of protein/ml was heated at 70 °C for 10 min to release bound nucleotides and P_i (Hanada *et al.*, 1989; Beharry & Bragg, 1991a). The supernatant (0.5 or 1.0 ml) was assayed for P_i. In some cases, 5 nmol of P_i was added before assay. At least two samples of each system were assayed. Both assays (with and without added P_i) gave the same values for P_i bound to the enzyme.

Preloading of F₁ with adenine nucleotides

F₁ was preloaded with adenine nucleotides and P_i by incubation of the enzyme for 30 s with 0.25 mM-ATP in 100 mM-Tris/acetate buffer, pH 6.8 (Kandpal *et al.*, 1987). Mg²⁺ was not added. The enzyme was then passed through a set of Sephadex G-50-80 centrifuged columns equilibrated with 100 mM-Tris/acetate buffer, pH 6.8. The enzyme was passed through a second set of columns if it was to be assayed for P_i. Non-preloaded F₁ was treated similarly.

ATP synthesis on F₁

The stock solutions, detailed protocol of experimental conditions for ATP synthesis, and the measurement of bound ATP and ADP by the luciferin-luciferase method, are described in Beharry & Bragg (1991a). The following modifications were made. When nucleotide was absent from the medium, three 100 µl samples from each 400 µl reaction mixture were desalted once only, and the volume of the combined column eluates was made up to 300 µl with the appropriate buffer, i.e. 100 mM-Tris/acetate, pH 6.8, with or without 30 % Me₂SO. Note that the stock solution of 100 mM-Tris/acetate buffer, pH 6.8, containing 40 % Me₂SO was made just before use, since the pH of this solution tends to fall if the solution is kept, even at 4 °C.

For measurement of bound P_i under ATP-synthesis conditions in the absence of exogenous ADP and P_i, three 125 µl samples of the 400 µl reaction mixture were desalted once only on Sephadex columns. Four reaction mixtures were incubated for each time point. The combined desalted eluates were sampled for P_i (1.1 ml), protein (25, 50 and 100 µl) and activity assays.

RESULTS

E. coli F₁ was analysed for the presence of endogenous bound adenine nucleotide and P_i. As shown in Table 1, the bound nucleotide content was 2.9 ± 0.1 mol/mol of F₁. An equivalent amount of P_i (3.1 ± 0.3 mol/mol of F₁) was present also.

Formation of ATP in Me₂SO

ATP synthesis by *E. coli* F₁ was examined at various concentrations of Me₂SO in the synthesis buffer (Fig. 1). Other conditions were set to optimize ATP formation. Formation of enzyme-bound [³²P]ATP from [³²P]P_i and ADP was maximal at 30–40 % Me₂SO and declined drastically both above and below this concentration range. Similar results were obtained if the enzyme had been concentrated by precipitation with (NH₄)₂SO₄ or by ultrafiltration.

The contents of bound ADP and ATP in F₁ incubated with exogenous ADP and P_i in aqueous and 30 % Me₂SO media were measured (Table 1). Almost 90 % of the bound adenine nucleotide was ADP. There was little change in the bound nucleotide components when the enzyme was transferred into 30 % Me₂SO in the absence of ADP and P_i. Incubation in the solvent with P_i

resulted in the conversion of endogenous ADP into ATP such that the nucleotide content of the enzyme was approx. 1 mol of ATP and 2 mol of ADP/mol of F₁. Addition of ADP together with P_i did not increase the amount of ATP formed, indicating that the ATP is synthesized solely from endogenous ADP. Since there is net formation of ATP in Me₂SO, the incorporation of [³²P]P_i into bound ATP shown in Fig. 1 is not due to exchange between the catalytic site and the medium. In the presence of exogenous ADP, a further molecule of ADP was bound, presumably at a non-catalytic site, in the Me₂SO system.

In the absence of Me₂SO, neither endogenous or exogenous ADP was converted into ATP. Almost two additional molecules of ADP were bound, as expected from the concentration of Mg²⁺ (4 mM) present (Lunardi *et al.*, 1981; Wise *et al.*, 1981). Thus one molecule less of ADP is bound to F₁ in the presence of 30 % Me₂SO.

Changes in the levels of bound nucleotide and P_i during incubation in Me₂SO

E. coli F₁ was incubated briefly with ATP to ensure maximum site occupancy (Kandpal *et al.*, 1987). The enzyme contained

Table 1. Adenine nucleotide content of non-preloaded F₁ incubated with ADP and P_i in the absence and presence of 30 % Me₂SO

The experiment was carried out as described in the Materials and methods section. Each value is the mean ± s.d. of values obtained from three separate experiments.

Solvent	Addition	Nucleotide bound (mol/mol of F ₁)		
		ATP	ADP	ATP+ADP
Aqueous	None	0.33 ± 0.12	2.6 ± 0.1	2.9 ± 0.1
	P _i (4 mM)	0.28 ± 0.06	2.6 ± 0.1	2.9 ± 0.1
	P _i , ADP (4 mM)	0.23 ± 0.06	4.3 ± 0.5	4.6 ± 0.6
Me ₂ SO	None	0.36 ± 0.10	2.2 ± 0.4	2.6 ± 0.4
	P _i	0.84 ± 0.15	1.9 ± 0.3	2.8 ± 0.2
	P _i , ADP	0.82 ± 0.12	2.7 ± 0.4	3.5 ± 0.3

Fig. 1. Synthesis of bound ATP at different concentrations of Me₂SO

F₁ (1.25 µM) in 0.1 M-Tris/acetate buffer, pH 6.8, was incubated in Me₂SO with 4 mM-MgCl₂ for 5 min at 30 °C. ADP (4 mM) and [³²P]P_i (4 mM; 2.5 × 10⁶ d.p.m./nmol) in Tris buffer were then added. After a further 5 min incubation, three 100 µl samples were freed of unbound ADP and P_i on centrifuged columns of Sephadex G-50-80 equilibrated with Tris/acetate buffer containing the appropriate concentration of Me₂SO. The column eluates were collected in 0.1 ml of 0.5 M-HClO₄ containing 10 mM-ATP, -ADP and -P_i. Denatured F₁ was removed by centrifugation. Duplicate 20 µl samples of the supernatants were applied to polyethyleneimine thin-layer plates for analysis of labelled nucleotides as described by Beharry & Gresser (1987).

Fig. 2. Changes in the concentrations of bound adenine nucleotides in nucleotide-preloaded F₁ after dilution into buffer containing 30% (v/v) Me₂SO

F₁ was preloaded with adenine nucleotide and the experiment was performed as described in the Materials and methods section. Total = ATP + ADP. The points are the means of at least three determinations. The vertical bars show the range of values obtained.

Fig. 3. Changes in the concentration of bound P_i in nucleotide-preloaded F₁ after dilution into buffer with and without 30% (v/v) Me₂SO

F₁ was preloaded with adenine nucleotide and the experiment was performed as described in the Materials and methods section. The points are the means of at least three determinations. The vertical bars show the range of values obtained.

about 5.6 mol of adenine nucleotide/mol of F₁ after incubation. The nucleotide was almost entirely ADP (Figs. 2 and 3). Analysis of the preloaded enzyme for P_i gave 6.0 ± 0.5 mol of bound P_i/mol of F₁.

Transfer of the preloaded F₁ into 30% -Me₂SO buffer in the

Fig. 4. Changes in the concentrations of bound adenine nucleotides in nucleotide-preloaded F₁ after dilution into a buffer containing 30% (v/v) Me₂SO and 10 mM-P_i

F₁ was preloaded with adenine nucleotide and the experiment was performed as described in the Materials and methods section. Total = ATP + ADP. The points are the means of at least three determinations. The vertical bars show the range of values obtained.

absence of exogenous P_i and adenine nucleotide resulted in the release within 5 min of 1.6 mol of ADP/mol of F₁ (Fig. 2). A portion of this ADP was subsequently re-bound. P_i was rapidly released also from the preloaded F₁ on transfer into Me₂SO buffer. Thus 2 mol of P_i/mol of F₁ was lost within 5 min. This was followed by the slower release of a further molecule of P_i. A small amount of ATP was formed on F₁ during the incubation, possibly by incorporation of some of the P_i released from the enzyme. Phosphate was not lost from preloaded F₁ if it was incubated in the absence of Me₂SO (Fig. 3).

Transfer of preloaded F₁ into 30% -Me₂SO buffer containing 10 mM-P_i again resulted in the release and re-binding of bound adenine nucleotides (Fig. 4). (The preloaded enzyme used to give the data shown in Fig. 4 contained more ATP than that used for the experiment shown in Fig. 2. However, the total nucleotide content was similar. We have found that there are small variations in the extent of conversion of ATP into ADP during preloading.) In contrast with the experiment of Fig. 2, about 3 mol of adenine nucleotide/mol of F₁ was released within 5 min in the presence of 10 mM-P_i. This was followed by re-binding of about 2 mol of adenine nucleotide/mol of F₁ and by the net synthesis of ATP (Fig. 4). The rate of ATP synthesis was slower than in the presence of exogenous ADP (e.g. Fig. 1; Table 1).

DISCUSSION

The F₁-ATPase of *E. coli* used in these experiments contained 3 mol of bound adenine nucleotide/mol of F₁ and 3 mol of bound P_i/mol of F₁. This is the first report of the presence of

bound P_i in preparations of *E. coli* F_1 , although recently we have found bound P_i in bovine heart mitochondrial F_1 (Beharry & Bragg, 1991b). The adenine nucleotide in our preparations was mainly ADP. This contrasts with our previous results (Bragg & Hou, 1977) and those of Maeda *et al.* (1976), in which the ATP/ADP ratio was 1–1.5:1, and also with the observation that in membrane-bound F_1 the ratio is 1.5–2:1 (Harris, 1978). It is likely that conversion of ATP into ADP occurred during the storage of F_1 as a suspension in $(NH_4)_2SO_4$ solution. The site occupancy of the *E. coli* F_1 is not known, but, by comparison with the mitochondrial enzyme (Kironde & Cross, 1986, 1987), is likely to be $F_1[2,1]$. Preincubation of this $F_1[2,1]$ enzyme with ATP resulted in the binding of nucleotide to give $F_1[3,3]$. Although Mg^{2+} was not added, hydrolysis of ATP occurred during preloading to give ADP. Thus the presence of 6 mol of P_i /mol of F_1 in the preloaded enzyme is consistent with the presence of ADP and P_i at all six binding sites. It is not clear at present if this implies that the unloaded enzyme is $F_1[3,0]$ or that loading of non-catalytic sites occurs after hydrolysis and release of nucleotide and P_i from catalytic sites.

Transfer of preloaded F_1 into 30% Me_2SO results in the release and re-binding of adenine nucleotide and the release of P_i . The process of release of adenine nucleotide is facilitated by the presence of 10 mM- P_i . The data of Figs. 2 and 4 taken together suggest that $F_1[3,3]$ is converted into $F_1[2,1]$ or $F_1[3,0]$ as the enzyme is converted from a conformation in which hydrolysis is favoured into a conformation favouring ATP synthesis. P_i (3 mol/mol of F_1) is released in the process. Adenine nucleotide re-binds to the enzyme in its new conformation to give $F_1[3,2]$, with a decrease in the affinity for nucleotide at one site. ATP is synthesized from bound ADP. Little ATP was synthesized in the absence of exogenous P_i . Presumably the affinity for phosphate at the catalytic site is diminished in the new conformation, and a higher concentration of exogenous P_i is necessary to re-bind significant amounts of phosphate for synthesis. This contrasts with our recent finding with bovine heart mitochondrial F_1 , where endogenous P_i is incorporated into ATP during synthesis in Me_2SO (Beharry & Bragg, 1991b). This result is due presumably to a difference in the affinity of the two F_1 -ATPases for P_i .

The results described above are consistent with the F_1 adopting a conformation in Me_2SO which favours ATP synthesis, as opposed to the conformation in entirely aqueous buffer, which favours ATP hydrolysis. We have shown, by chemical modification (Beharry & Bragg, 1989) and cross-linking (Beharry & Bragg, 1992), the existence of this conformational change in bovine heart mitochondrial F_1 . It is interesting that a somewhat analogous release and re-binding of adenine nucleotide, and an effect of P_i in the medium on this process, have been observed with mitochondrial and chloroplast ATPases on initiation of mitochondrial respiratory-chain activity or on photoactivation of chloroplasts (Rosing *et al.*, 1976; Strotmann *et al.*, 1976; Gräber *et al.*, 1977; Dunham & Selman, 1981; Penefsky, 1985; Fromme & Gräber, 1990). Thus the Me_2SO system should be an

ideal model in which to study conformational coupling to ATP synthesis.

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