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TIGHT BINDING OF ADENINE NUCLEOTIDES TO BEEF-HEART MITOCHONDRIAL ATPase

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SUMMARY

Mitochondrial ATPase (F_1) isolated from beef heart contains 5 moles of tightly bound adenine nucleotide per mole of enzyme—3 moles ATP and 2 moles ADP. These are retained after repeated precipitation of the enzyme with $(NH_4)_2SO_4$ and are only partially removed by treating the enzyme with activated charcoal or filtering through Sephadex. Cold denaturation, however, causes complete release of the adenine nucleotides from the protein. The exchange of the bound nucleotides with added nucleotides is slow.

Coupling factor 1 (F_1), a membrane-bound protein that can be isolated from mitochondria[1–3] and chloroplasts[4], is believed to be responsible for ATP synthesis coupled to electron transport in these organelles[5]. As such, its interactions with nucleotides and inorganic phosphate are of considerable interest.

F_1, when free from a naturally occurring inhibitor protein[6,7] or after trypsin treatment[8,4], has a very high ATPase activity in the presence of Mg^{2+} (or, in the case of chloroplast F_1, Ca^{2+}). In the absence of Mg^{2+} a single turnover of the enzyme is observed (Rosing, J., Harris, D. A. and Slater, E. C., unpublished observations). One binding site for added ADP has been found on rat-liver F_1[9], two on beef-heart F_1[10]. These sites are such that the bound ADP is rapidly exchangeable ($t_{1/2} < 10$ min) with added nucleotides. Chloroplast F_1 also contains two binding sites for ADP but the exchange is slower[11].

It is shown below that beef-heart F_1, after washing by repeated precipitation, contains non-covalently bound nucleotides and magnesium at sites with an affinity far higher than any preciously reported ($K_D < 0.1$ μM). The exchange of the bound nucleotides is correspondingly slow and they are not removed by any simple treatment (addition of activated charcoal or Sephadex filtration). In the light of these results, care must be taken in the interpretation of experiments involving the binding of labelled adenine nucleotides to F_1, particularly where relatively long periods of incubation and/or low concentrations of added nucleotides are involved.

F_1 was prepared by the method of Knowles and Penefsky[12]. Protein was estimated by the method of Lowry *et al.*[13] using bovine serum albumin as a standard.

The specific activity of the preparation was about 100 μmoles phosphate released per min per mg protein, measured in the presence of an ATP-regenerating system by the method of Pullman *et al.*[1]. The molecular weight of the enzyme was taken as 360 000[14].

The enzyme was washed as follows. The protein (approx. 6 mg/ml), dissolved in 0.25 M sucrose–10 mM Tris acetate–2 mM EDTA at pH 7.5, was precipitated by the addition of an equal volume of satd $(NH_4)_2SO_4$ solution (pH 7.2) and, after mixing, the suspension was allowed to stand in ice for 10 min. The protein was spun down at 0 °C, and the supernatant discarded. The dried pellet was carefully dissolved in the sucrose–Tris–EDTA medium at room temperature and, if necessary, clarified by centrifugation at room temperature.

For the estimations of bound adenine nucleotide and phosphate, the protein was washed 4 times by this procedure, or dialysed overnight against a 1000-fold excess of sucrose–Tris–EDTA medium and washed twice. This reduced the concentration of the supernatant adenine nucleotide, derived from the solution in which the enzyme was routinely stored, to a very low level (<0.1 μM) as checked by the use of radioactive ATP. The specific activity of the preparation fell by less than 10% during this procedure. A neutralized $HClO_4$ extract of the protein was then prepared as described by Rosing and Slater[15]. Adenine nucleotides were estimated enzymically in this extract by the methods of Bergmeyer[16] using an Aminco–Chance dual-wavelength spectrophotometer. Inorganic phosphate was estimated by the method of Wahler and Wollenberger[17].

Radioactivity was measured using a Nuclear Chicago liquid-scintillation counter type ISOCAP 300. As scintillation liquid was used a mixture of toluene and 96% ethanol (19:6, v/v) containing 4 g 2,5-diphenyloxazole and 50 mg 1,4-bis-(5-phenyloxazolyl-2-)benzene per litre. 10 ml of this mixture was added to a counting vial with 25 μl of the reaction mixture.

It was necessary to treat the radioactively labelled ATP solutions with phosphoenolpyruvate, pyruvate kinase and Mg^{2+} in order to eliminate contamination by traces of the carrier-free ADP. After this treatment an excess of EDTA was added to remove the free Mg^{2+}.

The results of the assays are given in Table I. Our preparation of F_1 contains up to 5 molecules of non-covalently but tightly bound adenine nucleotide per molecule of enzyme, 3 of ATP, and 2 of ADP. AMP and inorganic phosphate are absent. The nucleotides are released to the supernatant by cold denaturation of the enzyme, a process which involves separation of the enzyme into subunits[18]. This implies either that cold denaturation changes the tertiary structure of the subunits in such a way as to alter the binding, or that the nucleotides are bound at interfaces between the subunits. The bound nucleotides are removed to only a small extent from the enzyme by filtration through Sephadex G-25 (the small decrease seen here may be due to some denaturation of the enzyme, which was always observed during this procedure) or by shaking with activated charcoal, which did not affect the activity.

All the results quoted were obtained on F_1 prepared by DEAE-Sephadex filtration[12], but similar results were obtained on a sample prepared by protamine precipitation[19].

The enzyme contains 2 moles magnesium per mole enzyme (Yeates, R.A.,

TABLE I

TIGHTLY BOUND LIGANDS ON F_1

Values given are the range, in moles per mole enzyme, with the number of determinations in parentheses.

	ATP	*ADP*	*AMP*	P_i	Mg^{2+} *
Washed F_1	2.7–3.0 (4)	1.5–2.0 (4)	<0.1(4)	<0.05	2.2
Cold-denatured F_1[a]	<0.05	<0.05	<0.1	—	—
Sephadex-filtered F_1[b]	2.0–2.3 (3)	1.4 (2)	<0.1	—	—
Charcoal-treated F_1[c]	1.9–2.7 (3)	0.8–1.9 (3)	<0.1	—	—

* Yeates, R. A., personal communication.

[a] Cold-denatured F_1 was prepared from the 3-times washed protein by allowing a solution of 0.5 mg/ml F_1 to stand in 0.2 M KNO_3 at 0 °C for 2 h. The protein was then precipitated with $(NH_4)_2SO_4$ and washed once more. Cold denaturation did not affect the recovery of protein by the ammonium sulphate precipitation procedure. The denatured preparation had no ATPase activity.

[b] 3-times washed F_1 was filtered through a Sephadex G-25 (coarse) column (1m × 0.9 cm) at room temperature. The protein was applied in 0.5 ml of a 15 mg/ml solution in sucrose–Tris–EDTA medium. The eluate containing the F_1 was concentrated by $(NH_4)_2SO_4$ precipitation. A loss of about 20–30% of the specific activity was observed.

[c] 3-times washed F_1 was dissolved in sucrose–Tris–EDTA medium at a concentration of 1 mg/ml and the solution shaken gently for 5 min with 2 mg charcoal/mg F_1. The charcoal was removed by centrifugation and the protein precipitated with $(NH_4)_2SO_4$. The specific activity was unaltered by this procedure.

personal communication) although it is prepared in a magnesium-free environment, in the presence of 2 mM EDTA. These bound ions are not 'catalytically active' in the sense that added magnesium is necessary for continued ATP hydrolysis. However, Mg^{2+}–ATP is probably not the substrate so that there must be other binding sites for free Mg^{2+} on the enzyme. The relationship between the 'tight' and 'weak' sites for nucleotides (see above) and magnesium is that present under investigation.

In keeping with their tight binding, the bound nucleotides exchange with added nucleotides only very slowly, only about 30–40% of the bound ATP or ADP exchanging over a period of 24 h (Figs 1A and 1B). Magnesium stimulates exchange to only a small extent with ADP (not shown) and it inhibits the exchange with ATP probably by allowing hydrolysis to the more slowly exchanging ADP. The presence of 0.1 M KNO_3 also does not affect the exchange, although it apparently 'loosens' the protein structure as judged by its effect on the reactivity of sulphydryl groups and cold denaturation kinetics[18]. From the time course of the exchange it appears that one molecule of both ATP and ADP exchanges more rapidly than the others, but the reason for the non-exponential course of the ADP exchange, although always observed, remains uncertain. It may represent more than one stable conformation of the enzyme in our preparation.

In so far as these results may be extrapolated to the membrane-bound enzyme system, a pool of protein-bound nucleotides may exist in the mitochondrion (*cf.* Cross and Boyer[20]). It has been shown that in rat-liver mitochondria the inner-mitochondrial nucleotide pool is of the order of 10–15 nmoles/mg protein[21] and

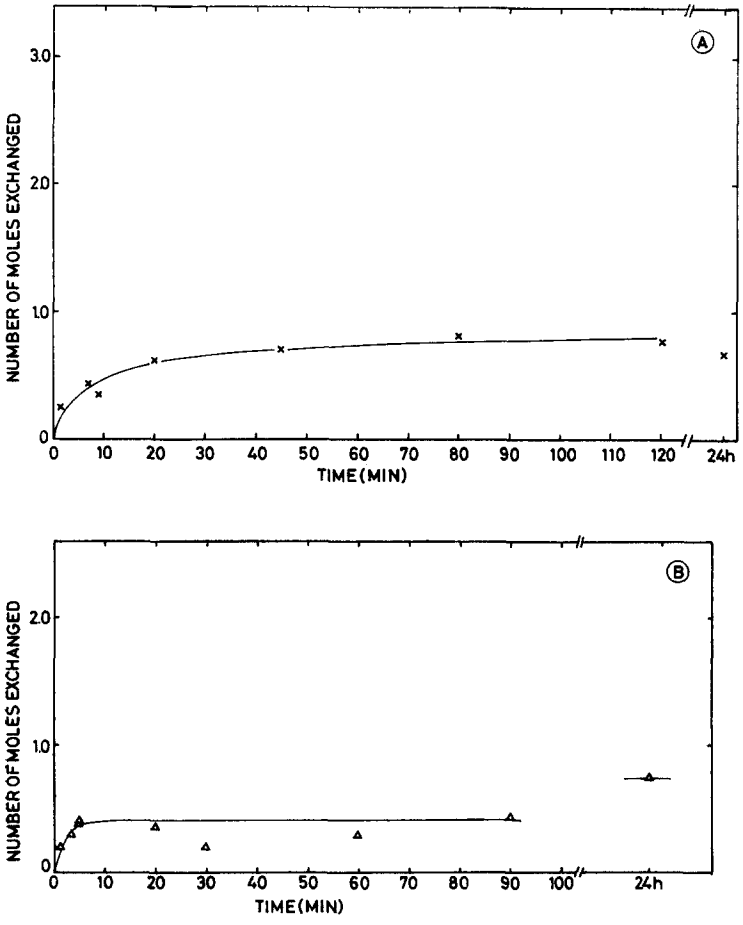

Fig. 1. Exchange of tightly bound nucleotides in the absence of Mg^{2+}. 0.1-ml aliquots (approx. 1 mg) of 4-times washed F_1 were incubated in the sucrose–Tris–EDTA medium containing 0.5 mM [^{3}H]ATP (A) or 0.5 mM [^{14}C]ADP (B). The protein was precipitated after various incubation times by the addition of 0.4 ml satd $(NH_4)_2SO_4$ solution and 0.2 ml sucrose–Tris–EDTA, and washed 4 times as described. After the fourth wash, the protein was redissolved in 0.1 ml sucrose–Tris–EDTA, the solution clarified by centrifugation, and aliquots taken for protein determination and radioactive counting, as described above.

that the F_1 concentration, calculated from aurovertin-binding data[22], is 0.12 nmole/mg protein. Therefore, if rat-liver F_1 binds nucleotides in a similar manner to beef-heart F_1, we can calculate that the bound nucleotides would form about 5% of the inner nucleotide pool. This value may well be higher in beef-heart mitochondria, which contain a smaller pool of nucleotides, and a larger concentration of F_1.

The selective tight binding of ATP in this way could provide a method for the synthesis of ATP, by altering the position of the $ATP+H_2O \rightleftharpoons ADP+P_i$ equilibrium (*cf.* ref. 23).

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