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BINDING OF ADP TO BEEF-HEART MITOCHONDRIAL ATPase (F_1)

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Summary

1. ADP binding to beef-heart mitochondrial ATPase (F_1), in the absence of Mg^{2+}, has been determined by separating the free ligand by ultrafiltration and determining it in the filtrate by a specially modified isotachophoretic procedure.

2. Since during the binding experiments the 'tightly' bound ADP (but not the ATP) dissociates, it is necessary to take this into account in calculating the binding parameters.

3. The binding data show that only one tight binding site (K_d about 0.5 μM) for ADP is present.

4. It is not possible to calculate from the binding data alone the number of or the dissociation constants for the weak binding sites. It can be concluded, however, that the latter is not less than about 50 μM.

Introduction

Several papers have appeared reporting the determination of the binding parameters for radioactively labelled ADP or ATP to mitochondrial ATPase F_1 [1—6]. However, most of these studies were made before it was found that isolated F_1 contains firmly bound ATP and ADP that exchange slowly and incompletely with added nucleotide [7,8], which makes it difficult to interpret the binding data. Since no satisfactory procedure could be developed to correct for this exchange, in this study the binding of ADP to F_1 has been determined directly by adding known amounts of unlabelled ADP to F_1,

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Abbreviations: F_1, mitochondrial ATPase.

separating the free ligand by ultrafiltration and determining it in the filtrate by isotachophoresis [9,10].

Methods

ATPase F_1

F_1 was prepared according to Knowles and Penefsky [11]. The preparation was stored in liquid N_2 in a solution containing 0.25 M sucrose, 10 mM Tris-acetic acid buffer (pH 7.5), 2 mM ATP and 2 mM EDTA. Prior to use, the protein was precipitated from the thawed solution by adding an equal volume of neutralized satd. $(NH_4)_2SO_4$, centrifuged and dissolved in the solution used in the binding experiments (see below). This washing procedure was twice repeated and the solution finally dialysed for 3 periods of 50 min each against 100 vols. of the solution used in the binding experiment. The residual concentration of $(NH_4)_2SO_4$ was 0.5 mM. Denatured protein was removed by centrifugation and the protein concentration determined by the Lowry method, using bovine serum albumin (A_{279nm}, 0.667 $cm^2 \cdot mg^{-1}$) as standard. The molecular weight was assumed to be 319 000 [12].

The specific activity of the ATPase, measured as described by Wagenvoord et al. [13], was 111 μmol/min per mg protein. Tightly bound nucleotides were determined in a neutralized perchloric acid extract as described by Harris et al. [7], employing pyruvate kinase to convert ADP to ATP and measuring the latter by the luciferase method.

ADP

ADP (free acid) from Boehringer contained less than 1% impurities detectable by isotachophoresis. The concentration of the stock solution was determined spectrophotometrically with pyruvate kinase and lactate dehydrogenase [14]. The concentration of dilutions of this stock solution that were added to the F_1 solution was calculated by the dilution factor and checked by isotachophoretic analysis. The values found agreed within 1—2% with the calculated value.

Ultrafiltration

A special ultrafiltration cell was constructed for these studies, designed for 0.3- to 5-ml volumes and with a small dead volume (50 μl) between membrane and filtrate output [15].

Before use, the membranes (Amicon Diaflo PM 10) were soaked in distilled water overnight to remove glycerol and azide. After mounting in the cell, the membrane was flushed with 1—2 ml distilled water (at 2 atm N_2 pressure) and with about 0.5 ml of the binding medium containing ADP at a concentration equal to that added to the binding medium. The filter was then removed and blotted with paper tissue on both sides, the liquid container of the cell wiped dry and the filtrate channel emptied by a stream of nitrogen.

After reassembling the cell, the F_1 solution and ADP solution were mixed and brought on to the cell, the total volume being 0.7 ml. After 5 min standing at 18°C, filtration was started with N_2 at 2 atm, and four filtrate fractions, each of about 30 μl, were collected in polythene tubes and immediately frozen

in liquid N_2 until analysis. Since the first filtrate fraction contained some ADP present in residual fluid (about 5 μl) in the filter, this was not used in the calculations. The amounts of ADP in the other three fractions did not differ appreciably, indicating that equilibrium was reached, and that ADP was not converted to other compounds during the ultrafiltration. The mean of the values for the second and third filtrates were used in the calculations.

Binding experiments

0.35 ml of a solution containing 0.25 M sucrose, 25 mM Tris-HCl buffer, 0.75 mM sodium acetate and 32 (Expt. 1) or 27.4 (Expt. 2) μM F_1 was mixed with 0.35 ml of a solution containing 0.25 M sucrose, 25 mM Tris-HCl buffer, 0.75 mM sodium acetate, 2 mM trisodium citrate and various amounts of ADP. The final pH of both solutions was 8.0. Demineralized and distilled (carbonate-free) water was used. All solutions were checked for the presence of anionic impurities by isotachophoresis. The citrate was added as a check that ions were not retained during ultrafiltration and as an internal standard to correct for concentration or dilution effects in handling the small sample volumes and for injection errors. Acetate was present as carrier for ADP in the isotachophoresis. EDTA was omitted since it binds to F_1 [15,16].

Isotachophoretic analysis

A new isotachophoretic procedure [9,10], using steady-state mixed zones, was developed for this investigation, since micromolar concentrations of ADP cannot be determined by usual isotachophoresis with 'pure' zones. A carrier and the operational system were chosen such that ADP and the carrier migrate

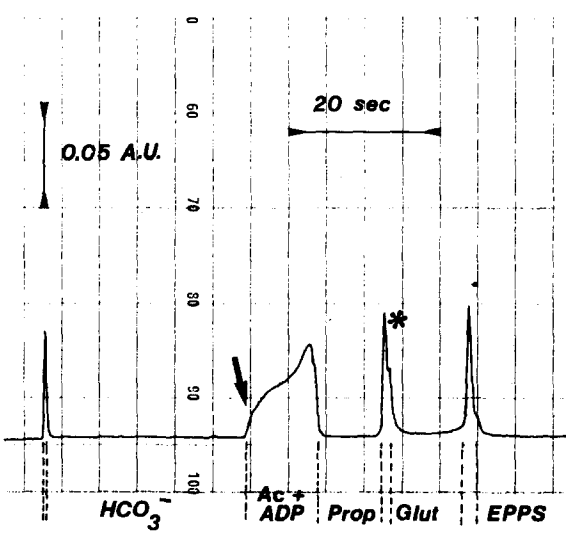

Fig. 1. Part of an isotachophoretogram (only absorbance signal is shown) of an ultrafiltrate sample from the experiment given in Fig. 3A. The arrow indicates where the presence of ATP would be noticed by the appearance of a spike. The spike with the asterisk enlarges in the presence of AMP. The ADP content of this sample was 38.4 pmol. Ac, acetate; Prop, propionate; Glut, glutamate; EPPS, 4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid.

with the same effective mobility thereby forming a steady-state mixed zone. Since ADP can be measured sensitively by ultraviolet spectrophotometry, it can be detected and measured in concentrations 2-3 orders of magnitude lower than that of other anions in the reaction mixture that appear in adjacent 'pure' zones.

Part of a typical isotachopherogram of an ultrafiltrate sample is shown in Fig. 1. Only the absorbance trace is shown (the isotachopherogram also records the electrical conductivity). The first and last spikes (from left to right) are due to minor impurities, the broad asymmetric 'peak' is due to ADP migrating together with acetate in the steady-state zone. The spike with the asterisk is partly due to AMP, present as a trace in the ADP preparation. Enlargement of this spike is an indication for AMP formation in the F_1 solution. The propionate and glutamate were not present in the filtrate sample but were injected separately. 4-(2-Hydroxyethyl)-1-piperazine propane sulfonic acid is the terminating ion. The amount of ADP is given by the area of the mixed zone. In this experiment, it is 38.4 pmol. If any ATP had been present (more than 5% of the ADP) it would have been detected by a spike at the position of the arrow. No ATP was detected in any of the filtrates from F_1 solutions, nor did the amount of AMP exceed that introduced with the ADP solution.

Results

Stirring artefact

Fig. 2 shows the results of a binding experiment when the solution above the membrane was stirred during ultrafiltration. It is clear that ADP is retained even in the absence of F_1, and there appears to be a large amount of non-specific binding. Other experiments [15] showed that EDTA is also retained, but there is little retention of Tris, whereas chlorate is excluded rather than retained.

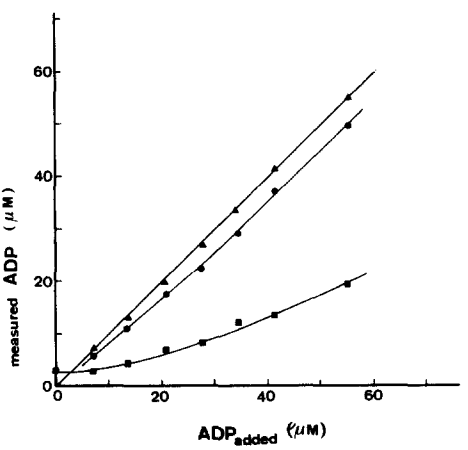

Fig. 2. Titration of F_1 with ADP in a stirred ultrafiltration experiment. ▲———▲, ADP determined in the binding mixture without F_1; ●———●, ADP in filtrate after blank filtration; ■———■, ADP in filtrate in presence of 14.2 μM F_1.

Table I shows that, in the absence of stirring, ions were not retained.

The cause of the stirring artefact [15] is not known. It is mentioned here in order to draw attention to a possible source of error, since stirring is often used in ultrafiltration cells.

Fig. 2 shows also that an appreciable amount of ADP was found in the filtrate even when none was added. This was unexpected, and it indicates that the 'firmly' bound ADP is less firmly bound than was believed, at least in this medium. In subsequent experiments, the amount of ADP present in the F_1 preparation was carefully determined and added to the amount of added ADP to give the total amount of ADP present. The amount found in the F_1 (0.7 mol/mol F) was appreciably less than 1 mol per mol F_1, presumably due to the extensive washing and dialysis in order to remove ATP present in the solution in which F_1 was stored.

Binding of ADP to F_1

In the lower curves of Figs. 3A and 3B is plotted the concentration of ADP in the ultrafiltrate for 10 and 8 different ultrafiltrations, respectively, with different amounts of ADP, plotted against the total amount of ADP in the solution before filtration, taking account of the amount of ADP initially present in the F_1. The straight line shows the amount of ADP measured in the absence of F_1 (open circles) or after a blank filtration (closed circles). This line coincides with the theoretical line with unit slope, assuming no retention in the filtrate in the absence of F_1. The difference between this line and the corresponding points representing free ADP in the ultrafiltrate in the presence of F_1 gives the amount of bound ADP. In Fig. 4 the amount of bound ADP is set out against the amount of free and in Fig. 5 the data are plotted in the form of Scatchard plots.

From both Fig. 3 and Fig. 4 it is clear that the binding of ADP is not homogeneous. Assuming that the F_1 preparation is homogeneous and that the molar concentration is correctly calculated, one molecule of ADP binds much more strongly than subsequent molecules. It is not possible directly from these experiments to determine the number of weak binding sites and their respective affinities. However, additional information may be used for the selection of a most probable binding model (see Discussion). The fitting of binding models to the data must take the analytical errors into account. A numerical procedure

TABLE I

TEST OF THE PASSAGE OF IONIC COMPONENTS THROUGH THE MEMBRANE

The test solutions contained 3.9 μM F_1 in 250 mM sucrose, 25 mM Tris/4-(2-hydroxyethyl)1-piperazine propane sulfonic acid buffer, 1.5 mM sodium acetate, 3 mM sodium chlorate and the specified anions (sodium salts) in the concentrations given. The final pH of these solutions was 8.0. In the absence of stirring, filtrations were performed as described under Methods. Quantitative analysis was done by isotachophoresis.

Component	EDTA	PP_i/EDTA	P_i/EDTA	Citrate/EDTA
In cell (mM)	0.74	0.41/1.75	1.27/1.80	0.89/1.51
In filtrate (mM)	0.74	0.42/1.72	1.24/1.74	0.90/1.52

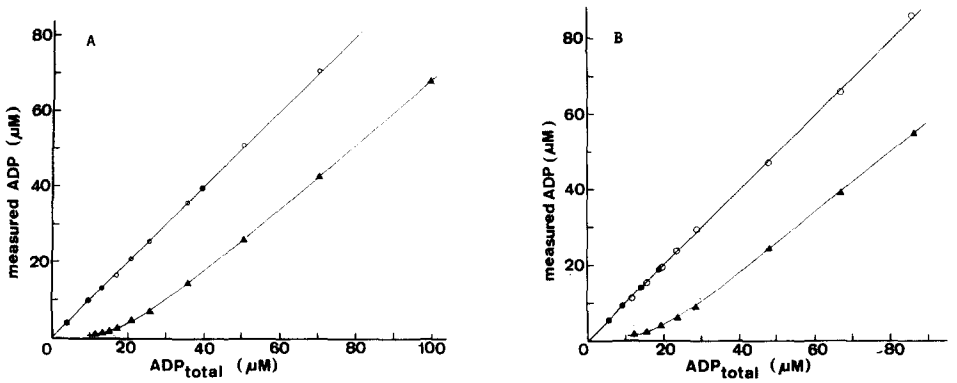

Fig. 3. Titration of F_1 with ADP when filtration was performed without stirring. ○——○, ADP determined in the binding mixture without F_1; ●——●, ADP determined in blank filtration without F_1; ▲——▲, ADP determined in filtrate with 16 μM (A) or 13.7 μM (B) F_1 present.

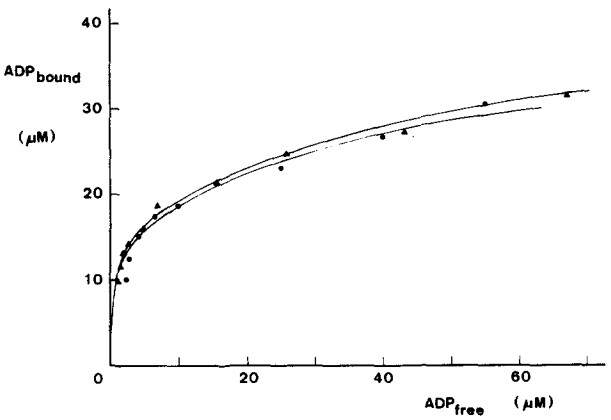

Fig. 4. Representation of data in Fig. 3A (▲——▲) and 3B (●——●) by plotting the ADP_{bound} as function of the ADP_{free}. The curves are calculated from the data given in Table II for a model with one strong and two weak (identical) sites.

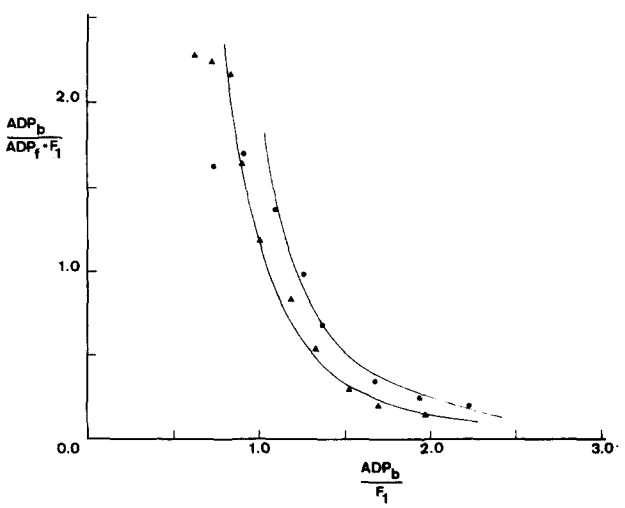

Fig. 5. Representation of the data of experiments given in Fig. 3A (▲——▲) and 3B (●——●) as a Scatchard plot. The curves are constructed from the data given in Table II for the model with one strong and two weak (identical) sites. The data for the lowest ADP_{bound} are not taken into account.

TABLE II

DISSOCIATION CONSTANTS OF ADP BOUND TO F_1 IN THE ABSENCE OF Mg^{2+}

K_{d_1} is the dissociation constant of ADP bound to the single strong site, K_{d_2} to the weak sites. Two calculations have been made assuming 2 and 4 homogeneous weak sites, respectively.

Experiment	2 weak sites (model A)		4 weak sites (model B)	
	K_{d_1} (μM)	K_{d_2} (μM)	K_{d_1} (μM)	K_{d_2} (μM)
1	0.61 ± 0.06 *	65 ± 5	0.54 ± 0.07	162 ± 15
2	0.31 ± 0.11	41 ± 3	0.09 ± 0.12	114 ± 11

* Estimated standard deviation.

that can effectively handle the error structure in the concentration of free and bound ligand has been developed by Linssen [17,18]. This program, which is suitable for non-linear regression with mutually dependent parameters, such as the free and bound ligand, has been used to determine the binding affinities presented in Table II. The fitting function used can be written as

$$\frac{[B]}{[F]} = \sum_i \left(\frac{n_i \cdot [E]}{K_{d_i} + [F]} \right)$$

where B is the bound ADP, F the free ADP and E the enzyme, n_i the number of sites (of kind i) per enzyme molecule and K_d the intrinsic dissociation constant. The error in the K_d values presented is calculated from the error in the determination of the concentrations bound and free. Other sources or error are briefly considered in the discussion.

The drawn lines in Figs. 3—5 are calculated on the assumption that F_1 contains one strong binding site and two weak sites with equal intrinsic binding constants (see Discussion).

Discussion

The simplest model fitting the binding data consists of one relatively strong site (K_d about 1 μM) and an infinite number of weak aspecific binding sites [15]. Although aspecific binding may well be taking place, other data indicate that specific ADP-binding sites are, in any case, present (see, e.g. Refs. 19 and 20). Moreover, a control experiment with bovine serum albumin showed that ADP does not bind aspecifically to proteins in general. No significant binding could be detected when up to 82 μM ADP was incubated with 46 μM albumin, under the same conditions as in the experiments with F_1.

In considering other possible binding models, the following properties of F_1 were taken into account. In most respects it behaves as a dimer $(\alpha\beta\gamma\delta\epsilon)_2$. One molecule contains 2 molecules tightly bound ATP [7], binds two molecules of aurovertin [12] (each to a β subunit), and is inhibited when two molecules of azido-ATP or azido-ADP are covalently bound, either to the α or β subunits [13,21]. Exceptions are inhibition by 4-chloro-7-nitrobenzofurazan chloride [22], which requires only one molecule bound to the β subunit, and the single tightly bound ADP (see below).

Wagenvoord et al. [13,21], have shown that, in the absence of Mg^{2+}, azido-ADP is bound equally strongly to two sites on F_1 (already containing one molecule of firmly bound ADP), one on an α subunit and one on a β subunit. Although binding of 2 molecules of azido-ADP is sufficient completely to inhibit the ATPase, it is likely that prolonged treatment with azido-ADP would eventually label both α and β subunits. In the absence of Mg^{2+} one molecule of azido-ATP is bound to each of the two β subunits and ADP prevents this binding, showing that the latter also binds to the β subunit. Two molecules of azido-ADP are also bound in the presence of Mg^{2+}, but now only to the two α subunits. ADP also prevents this binding. When the β subunits are first labelled with azido-ATP, in the absence of Mg^{2+}, 2 molecules of azido-ADP become bound to the α subunit in the presence of Mg^{2+}. The amount of firmly bound ATP and ADP is unaltered by these treatments. (Wagenvoord, R.J., van der Kraan, I. and Kemp, A., unpublished). Thus, it is very likely that there exist at least two and possibly four weak binding sites for ADP under the conditions of the experiments described in this paper.

No uncertainty exists concerning the number of tight ADP-binding sites. The binding data could be fitted only to models with a single tight site.

Although the uncertainty in the number of weak sites precludes an unambiguous calculation of the dissociation constant of ligand bound to these sites, calculations were made in order to obtain an idea of the order of magnitude of this constant. This is of interest in connection with the study of inhibition by ADP of the ATPase activity of F_1 [23]. The following assumptions were made: the F_1 preparation is homogeneous, the F_1 concentration has been calculated correctly, the binding sites are fully accessible and are mutually independent, and the bound and added ADP function as a single pool. Calculations have been carried out for a model (A) with 1 strong and 2 weak sites and for a second (B) with 1 strong and 4 weak sites. In both models the weak sites are considered equivalent. The results are given in Table II. It must be mentioned here that in these models a relatively small error in the F_1 concentration will lead to a large error in the affinity constants. For example, by using a molecular weight of 347 000 instead of 319 000, the K_d values are decreased by 52% and 27% for the strong and the weak sites, respectively, in model A (Expt. 2).

The dissociation constant of ADP bound to the strong sites is rather higher than had been believed, but it is consistent with the finding that repeated washing and dialysis of F_1 removes a considerable amount of this 'tightly bound' ADP. Which subunit bears this single strong site is not known.

Experiments with photoaffinity labels have shown that weak ADP-binding sites are present on the α and β subunits. Azido-ADP-, and presumably ADP-, binding sites are present on both α and β subunits, but it is not known if ADP, in the absence of Mg^{2+} and EDTA, binds to both α and β subunits or to only one of each. In the former case (model B) the calculated dissociation constants (assuming equal affinities of the sites) are 114 and 162 μM in the two experiments. If only two sites can be occupied (model A) the dissociation constants are 41 and 65 μM, respectively. Whereas the photoaffinity labelling favours the former possibility, a better fit to the binding data judged from the residual sums of squares was obtained with model A. It does seem reasonable to con-

clude from the data presented in Table II that there is a single binding site with a dissociation constant of about 0.5 μM and additional binding sites with dissociation constant of minimally about 50 μM.

Since no ATP was detected in the ultrafiltrates, exchange of ADP for tightly bound ATP, observed by Harris et al. [24] did not take place in our experiments. This may be due to both the high pH and the relatively low concentrations of ADP used in our experiments.

Tight and weak sites of ADP binding were previously observed by Hilborn and Hammes [3], who reported one strong site and one weak. The dissociation constants were 0.28 and 47 μM, respectively, in the presence of Mg^{2+} and 11 and 43 μM, respectively, in its absence. Strangely, our values obtained in the absence of Mg^{2+} agree fairly closely with theirs obtained in its presence, except for the number of weak sites, but not in its absence. Catterall and Pedersen [2] also found a single firmly bound ADP (K_d 0.9 μM) in liver F_1, in the absence of Mg^{2+}. Since ADP already bound to the F_1 was not taken into account in these studies, the K_d values may be under-estimated, especially for the strong sites.

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