Biochimica et Biophysica Acta, 376 (1975) 13–26

BBA 46858

NUCLEOTIDE-BINDING PROPERTIES OF NATIVE AND COLD-TREATED MITOCHONDRIAL ATPase

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(Received July 23rd, 1974)

SUMMARY

1. The bound nucleotides of the beef-heart mitochondrial ATPase (F_1) are lost during cold inactivation followed by $(NH_4)_2SO_4$ precipitation. The release of tightly bound ATP parallels the loss of ATPase activity during this process.

2. During cold inactivation, the sedimentation coefficient ($s_{20,w}$) of the ATPase first declines from 12.1 S to 9 S, then to 3.5 S. $(NH_4)_2SO_4$ precipitation of the 9-S component also leads to dissociation into subunits with $s_{20,w}$ of 3.5 S.

3. The 9-S component still contains the bound nucleotides, which are removed when it dissociates into smaller subunits.

4. Reactivation of cold-inactivated ATPase by incubation at 30 °C is increased by the presence of 25 % glycerol. ATP, however, does not have any clearcut effect on the degree of reactivation in the presence of glycerol.

5. ADP is an inhibitor of the reactivation, probably because it exchanges during reactivation for bound ATP giving rise to an inactive 12-S component.

6. The exchange of tightly bound nucleotides with added adenine nucleotides is more extensive and faster with cold-inactivated ATPase than with the native enzyme. During reactivation up to 1.6 moles of ATP and 1.0 mole ADP can exchange per mole enzyme.

7. Incubation with GTP, CTP or inorganic pyrophosphate induces an increased activity of the ATPase, which, however, soon declines in the presence of ATP. It also disappears on precipitation of GTP-treated enzyme with $(NH_4)_2SO_4$.

INTRODUCTION

Beef-heart mitochondrial ATPase (F_1) [1] and the corresponding enzymes isolated from chloroplast [2] and *Escherichia coli* membranes [3] are cold-labile enzymes, losing activity when kept at 0 °C. Penefsky and Warner [4] have shown

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that the inactivation follows first-order kinetics at low protein concentration and that it is accelerated by the presence of chaotropic salts. During cold inactivation the active enzyme (11.9 S) is transformed into a mixture of components with sedimentation coefficients of 11.9, 9.1 and 3.5 S. It is possible to reactivate the cold-treated enzyme and to re-form the 11.9-S component by incubation at 30 °C. Restoration of the coupling and Ca^{2+}-dependent ATPase activities of cold-treated chloroplast ATPase (CF_1) after cold inactivation is maximal in the presence of ATP and 25 % glycerol [5]. Penefsky and Warner used only ATP in their experiments.

We have previously reported that the isolated and purified mitochondrial ATPase contains three molecules of tightly bound ATP and two of tightly bound ADP per molecule of the enzyme, and that the nucleotides are released during cold inactivation of the enzyme followed by $(NH_4)_2SO_4$ precipitation [6, 7]. Abrams et al. [8] found 1 mole of tightly bound ATP and one of ADP in purified *Streptococcus faecalis* ATPase. It appears, then, that the tight binding of the adenine nucleotides is a property of the quaternary structure of the protein. In this paper, the relationship between the protein–nucleotide interactions and the intersubunit interactions in the protein, modified by cold treatment, have been further studied. It is shown that the nucleotide-binding properties are different for each of the species seen in the ultracentrifuge. A preliminary report of this work has been published elsewhere [9].

METHODS

Mitochondrial ATPase (F_1) was prepared by the method of Knowles and Penefsky [10]. Protein was estimated by the method of Lowry et al. [11] using bovine serum or egg albumin as a standard. The absorbance coefficient of these proteins at 280 nm was taken as 0.895 $(mg/ml)^{-1} \cdot cm^{-1}$. The specific activity directly after 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 protein was taken to be 360 000 [12].

Since the enzyme is stored in a buffer containing 0.25 M sucrose, 10 mM Tris acetate, 2 mM EDTA and 4 mM ATP at pH 7.5, it was freed from ATP by precipitation by addition of an equal volume of satd $(NH_4)_2SO_4$ solution (pH 7.2). After standing in ice for 10 min, the protein was spun down at 0 °C, the supernatant discarded and the dried pellet was carefully dissolved in the sucrose–Tris–EDTA medium at room temparature. The preparations used in the experiments described in this paper were washed 4 times by this procedure and if necessary finally clarified by centrifugation at room temperature. It was possible, by taking care to avoid cold or surface denaturation during this procedure, to restrict the loss in specific activity to less than 10 %.

For the estimations of bound nucleotides a neutralized $HClO_4$ extract of the protein was prepared as described by Rosing and Slater [13], and adenine nucleotides were estimated enzymatically in this extract by the methods of Bergmeyer for ATP [14] and ADP [15] using an Aminco Chance dual-wavelength spectrophotometer. In some experiments ATP was estimated by the luciferase method using a Unilux liquid scintillation counter [16].

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.

The radioactively labelled ATP solutions were treated with phosphoenol-pyruvate, 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 ATPase activity was measured with an ATP-regenerating system at 30 °C. The protein was first diluted in the sucrose–Tris-EDTA medium described above to a protein concentration of about 0.2 mg/ml. An aliquot of this solution containing about 2 μg protein was added to a cuvet containing 33 mM Tris acetate, 83 mM sucrose, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM ATP, 1.5 mM phosphoenolpyruvate, 0.17 mM NADH, 6 units (μmole/min) pyruvate kinase (EC 2. 7. 1. 40) and 12 units lactate dehydrogenase (EC 1.1.1.27) at pH 7.2. ATPase activity was calculated from the rate of NADH oxidation followed at 340 nm in a Zeiss spectrophotometer. The activity of the washed enzyme measured by this method was usually less than 100 μmoles/min per mg protein, the activity given above for the freshly prepared enzyme. This lower activity is due to the lower pH (7.2 instead of 7.5) at which the activity was measured and to some inactivation during washing of the ATPase.

Ultracentrifugation was performed in an MSE analytical ultracentrifuge, equipped with a 4-hole rotor using a single-sector (Fig. 8, 11A) or a double-sector cell (Fig. 11B, C) both with wedge windows. Viscometry at 20.0 °C. was carried out with a rotating-cylinder viscometer designed by Zimm and Crothers [17].

RESULTS

Reactivation of the enzyme after cold denaturation.

Fig. 1 gives the results of a typical cold inactivation–reactivation experiment. The inactivation was carried out at 0 °C and pH 7.5 in the presence of KNO_3 and the reactivation at 30 °C. In agreement with Lien et al. [5], who studied the chloroplast enzyme, glycerol clearly increases the degree of reactivation of the beef-heart enzyme. In agreement with Penefsky and Warner [4], it was found in other experiments that the degree of renaturation depends upon the extent of previous inactivation and on the protein concentration. The optimum protein concentration in the presence of glycerol (which appears to slow down non-specific aggregation and precipitation of the protein) was 1–2 mg/ml.

Unlike the chloroplast enzyme, however, the reactivation of beef-heart ATPase in the presence of glycerol is not greatly affected by the presence of ATP, even if all traces of free nucleotides are removed from the solution by charcoal after inactivation (Fig. 2).

Fig. 3 shows the effect of other nucleotides and of pyrophosphate on the reactivation. GTP, CTP (Fig. 3A) and pyrophosphate (Fig. 3B) appear to promote reactivation. The rate of increase of the ATPase activity in the presence of GTP and CTP is, however, clearly biphasic, a rapid phase being followed by a slow one. The increase of activity during the first rapid phase is about the same as that obtained

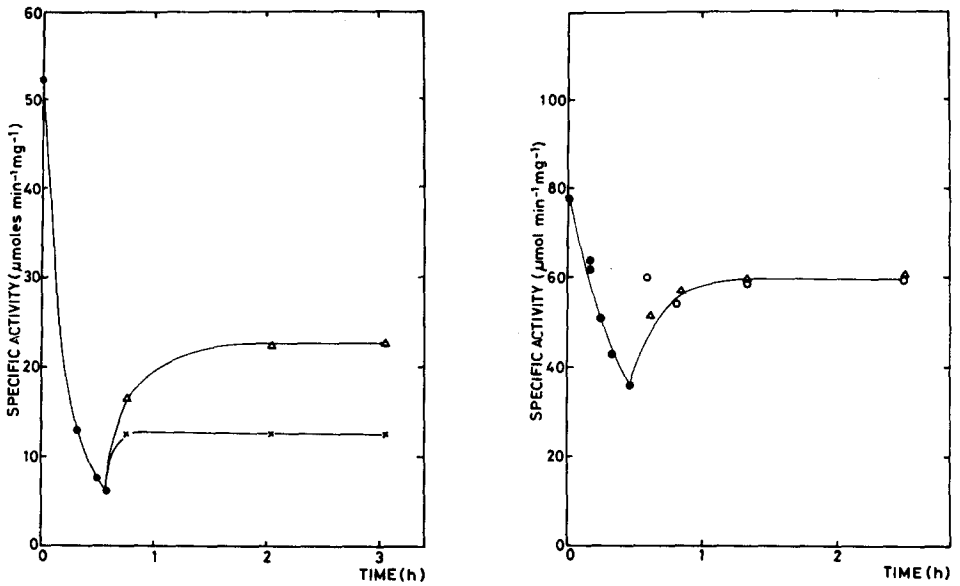


Fig. 1. Cold inactivation and renaturation of beef-heart ATPase. Washed ATPase was inactivated at 0 °C in a buffer containing 10 mM Tris maleate and 50 mM KNO_3 at pH 7.5, at a protein concentration of 2.0 mg/ml. After 35 min, reactivation was started by diluting 0.2-ml aliquots with 0.6 ml of a buffer containing 40 mM Tricine–NaOH and 4 mM ATP with (△) or without (×) 34 % (w/v) glycerol at pH 7.8. The solution was incubated at 30 °C throughout the remainder of the experiment.

Fig. 2. Effect of ATP on the reactivation of cold-inactivated ATPase. Washed ATPase was inactivated at 0 °C in a buffer containing 10 mM Tris maleate and 50 mM KNO_3 at pH 7.5, at a protein concentration of 4 mg/ml. After 30 min the solution was shaken with charcoal (20 mg/ml) at 0 °C to remove free ATP. After removal of the charcoal by centrifugation, 0.3-ml aliquots were mixed with 0.9 ml of a buffer containing 40 mM Tricine–NaOH and 34 % (w/v) glycerol at pH 7.8 with (△) or without (○) 4 mM ATP. The samples were reactivated at 30 °C.

in the presence of ATP. Unlike enzyme renatured in the presence of ATP, the ATPase activity of enzyme renatured in the presence of these compounds declines during measurement of the activity (cf. Fig. 3B).

These results suggest that the increased activity seen with GTP, CTP and PP_i in Fig. 3 is not due to an effect on the reactivation process, but that these compounds bind slowly to the ATPase, causing an increased activity. Indeed, if the untreated enzyme is incubated with GTP or PP_i at room temperature, its initial ATPase activity rises (Table I), again falling to the normal level with time. The ATPase–GTP complex, whether prepared from cold-denatured (see Table II, Expt C) or native enzyme (Table I) is not stable to $(NH_4)_2SO_4$ precipitation of the enzyme.

ADP clearly decreases the extent of reactivation (Fig. 3B). This effect is quite specific for ADP; GDP and IDP have no effect (not shown). Nucleotide analogues such as dATP (Fig. 3A) and adenosine, tripolyphosphate and β,γ-methylene ATP (not shown) also had little effect on the reactivation.

The only other treatment that was found to inhibit the reactivation was precipitation of the enzyme, after inactivation but prior to reactivation, by addition of $(NH_4)_2SO_4$ solution (Fig. 4). We have shown previously that intact and cold-inactivated ATPase are precipitated to the same extent by the $(NH_4)_2SO_4$ treatment [6].

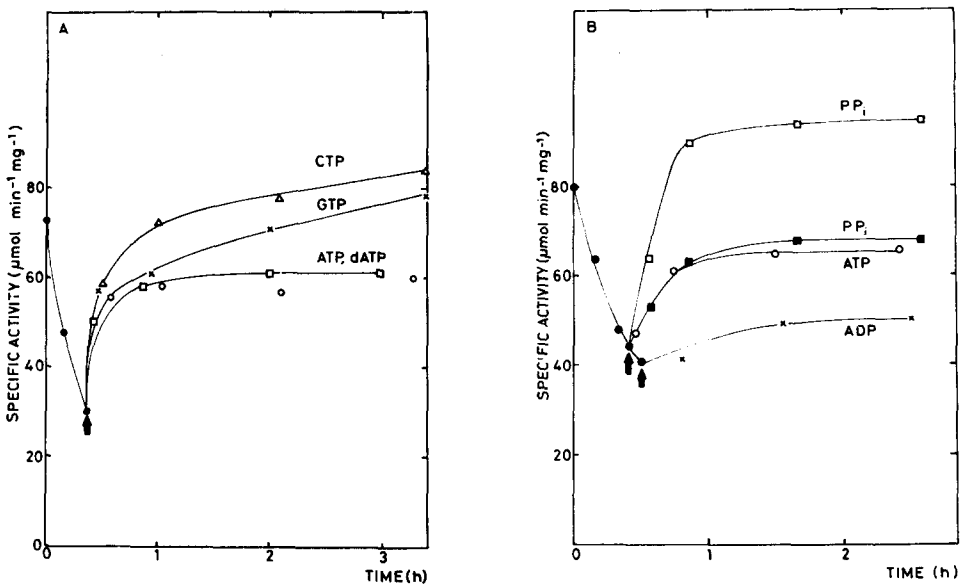


Fig. 3. Effect of GTP, CTP, ADP, pyrophosphate and dATP on the reactivation of cold-inactivated ATPase. Washed ATPase was inactivated at 0 °C in a buffer containing 10 mM Tris maleate and 50 mM KNO_3 at pH 7.5, at a protein concentration of 3.1 mg/ml. At the times indicated by arrows in the figures aliquots of 0.2 ml (A) or 0.3 ml (B) were mixed with 0.6 ml or 0.9 ml, respectively, of a buffer containing 40 mM Tricine–NaOH and 34% (w/v) glycerol at pH 7.8 and, in Fig. 3A, 4 mM ATP (□); 4 mM GTP (×); 4 mM CTP (△); or 4 mM dATP (○); and, in Fig. 3B, 4 mM ATP (○); 4 mM ADP (×); or 4 mm PP_i (□ and ■). The samples were reactivated at 30 °C. After reactivation in the presence of pyrophosphate, the ATPase activity declines to a constant value during the measurement. □, initial activity; ■, activity after 1–2 min.

TABLE I

EFFECT OF GTP ON THE ACTIVITY OF NATIVE ATPase

1.3 mg/ml washed ATPase (activity 95 μmoles/min per mg protein) was kept at 30 °C in the presence of 4 mM ATP or 4 mM GTP for different periods of time. At intervals samples were taken and the ATPase activity was measured. The activity of the ATPase kept in the presence of ATP was constant in time while that of the GTP-treated enzyme declined during the ATPase measurement. After 150 min the protein in both samples was precipitated by addition of an equal volume of satd $(NH_4)_2SO_4$ solution. The precipitated protein was spun down and dissolved in sucrose–Tris–EDTA buffer and the specific activity was again measured.

Time (min)	Specific activity of F_1 kept in ATP	Specific activity of F_1 kept in GTP	
		Initial	After 2 min
45	106	111	103
90	108	124	112
120	115	130	—
150	97	132	117
150*	106	119	106

* After $(NH_4)_2SO_4$ precipitation.

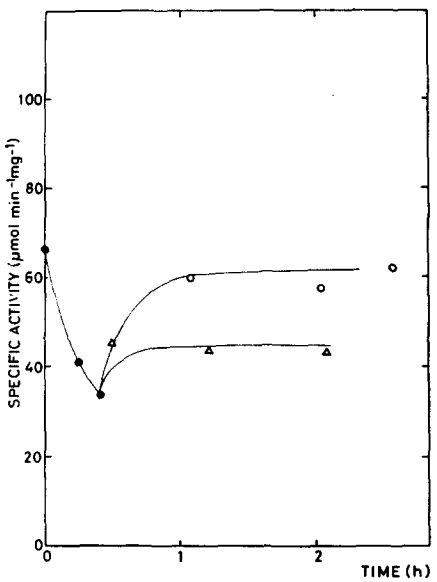


Fig. 4. Reactivation of ATPase after $(NH_4)_2SO_4$ precipitation of cold-inactivated enzyme. Washed ATPase was inactivated at 0 °C in a buffer containing 10 mM Tris maleate and 50 mM KNO_3 (pH 7.5), at a protein concentration of 4.3 mg/ml. After 25 min two samples were taken. The protein from one sample was precipitated immediately by addition of an equal volume of satd $(NH_4)_2SO_4$ solution. The precipitated protein was spun down and dissolved in 10 mM Tris maleate and 50 mM KNO_3 (pH 7.5) at room temperature. Samples of the non-precipitated and the precipitated cold-inactivated enzyme were diluted with 3 vol. of a buffer containing 40 mM Tricine–NaOH, 4 mM ATP and 34 % (w/v) glycerol at pH 7.8 and incubated at 30 °C. ○, non-precipitated; △, precipitated.

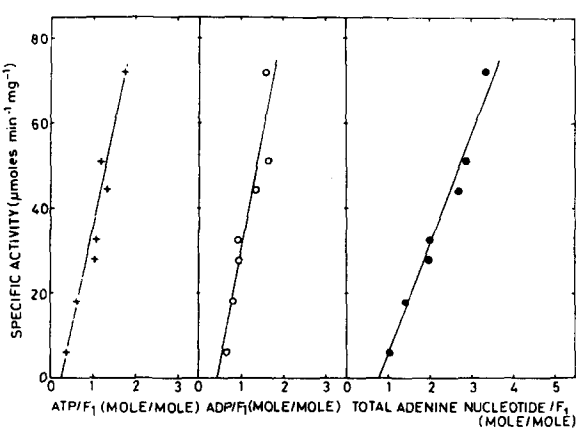


Fig. 5. Effect of cold inactivation on concentration of firmly bound adenine nucleotides in ATPase. Washed ATPase was inactivated at 0 °C in a buffer containing 20 mM Tris maleate and 50 mM KNO_3 at pH 7.5, at a protein concentration of about 2 mg/ml. At intervals the activity was measured and samples containing about 1 mg protein were taken and the protein precipitated by addition of an equal volume of satd $(NH_4)_2SO_4$ solution. The precipitated protein was spun down and redissolved in a buffer containing 0.25 M sucrose, 10 mM Tris acetate and 2 mM EDTA at pH 7.5. The ATPase activity of this solution was measured. The ATP and ADP contents were determined on an $HClO_4$ extract using the luciferase and pyruvate kinase–lactate dehydrogenase methods, respectively.

Bound-nucleotide content of ATPase during cold inactivation and reactivation

The bound nucleotides of beef-heart mitochondrial ATPase are released after complete acid denaturation of the enzyme [6]. The effect of cold inactivation on the amount of firmly bound nucleotide was followed by taking a sample at various times, precipitating the enzyme with 50 % $(NH_4)_2SO_4$ and measuring the activity and nucleotide content of the precipitate. Precipitation of cold-inactivated enzyme has little effect on the residual activity of the enzyme. Fig. 5 shows that during cold inactivation the bound nucleotides, measured in this way, declined parallel to the loss of ATPase activity.

Although ADP seems to bind somewhat more strongly to the enzyme (45 % of the ADP left at 20 % activity as compared to 25 % of the ATP), the behaviour of the

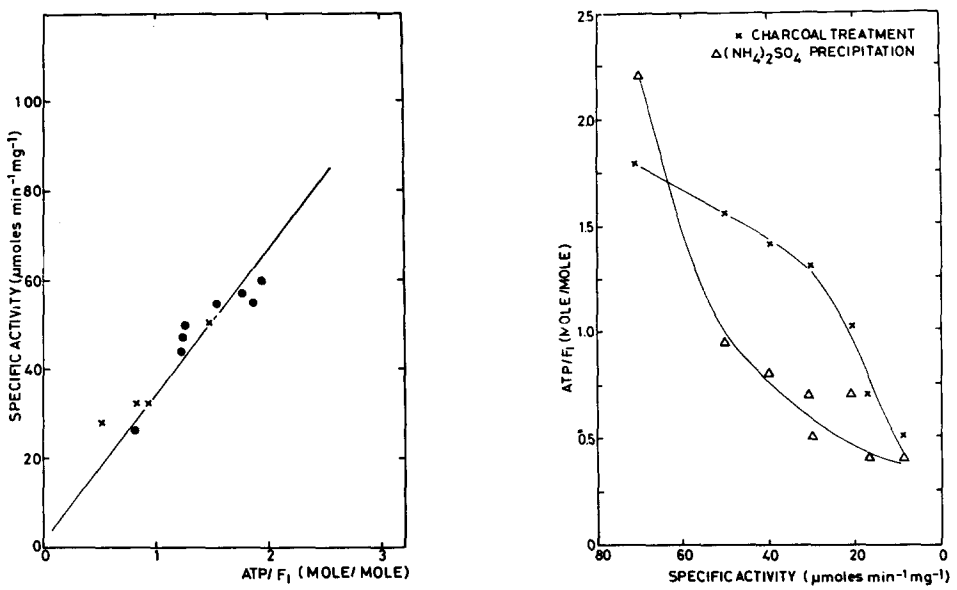


Fig. 6. Restoration of tight ATP binding during reactivation of cold-inactivated ATPase. Washed ATPase was inactivated and reactivated in a buffer containing 40 mM Tricine–NaOH and 34 % (w/v) glycerol as described in Fig. 3. The reactivation was carried out in the presence (●) and absence (×) of 1 mM ATP. At intervals samples containing about 1 mg protein were taken and the protein precipitated with an equal volume of satd $(NH_4)_2SO_4$ solution. The precipitated protein was spun down and redissolved in a buffer containing 0.25 M sucrose, 10 mM Tris acetate and 2 mM EDTA at pH 7.5. This washing procedure was repeated three times. The final precipitate was dissolved in sucrose–Tris–EDTA and clarified by centrifugation. The ATPase activity was determined on this solution and the ATP content on an $HClO_4$ extract using luciferase.

Fig. 7. The amount of bound ATP as a function of the specific activity of cold-inactivated F_1, precipitated with $(NH_4)_2SO_4$ or treated with charcoal. Washed ATPase was dissolved in a buffer containing 10 mM Tris maleate and 50 mM KNO_3 at pH 7.5 and inactivated at 0 °C, at a protein concentration of 1.5 mg/ml. At intervals the activity was measured and two samples were taken, one of which (×) was treated with charcoal (about 20 mg/ml sample). The charcoal was removed from the solution by centrifugation. In the other (△), the protein was precipitated by addition of an equal volume of satd $(NH_4)_2SO_4$ solution. The precipitated protein was spun down and redissolved in sucrose–Tris–EDTA buffer. The ATP content was determined on an $HClO_4$ extract of both solutions by the luciferase method.

two nucleotides is otherwise very similar. This stronger binding of ADP is also demonstrated when the enzyme is dialysed at room temperature in the absence of ATP. In 18 h we find a loss of 1 mole ATP per mole enzyme and 30 % of the activity, without any change in the ADP content. In the following experiments only the ATP content of the enzyme was determined since the greater sensitivity of the luciferase method of determining ATP permitted the use of smaller amounts of enzyme.

As shown in Fig. 6, the ATP content of the precipitated enzyme increases parallel with the ATPase activity even when no ATP is added during the reactivation. It appears, then, that the nucleotides are not released from the enzyme into solution during denaturation, but remain bound to the enzyme. However, in contrast to the behaviour of the native enzyme, the nucleotides are released by precipitation of the cold-treated protein with $(NH_4)_2SO_4$. This conclusion is supported by the experiment shown in Fig. 7. During the course of cold inactivation the specific activity was followed and at various times two samples were taken. One was treated with charcoal to remove free nucleotides from solution and the other was precipitated with $(NH_4)_2SO_4$ and redissolved in buffer. The ATP content of acid extracts of both samples was determined by the luciferase method. From Fig. 7 it is clear that more ATP remains bound to the protein after treatment with charcoal than after $(NH_4)_2SO_4$ precipitation. In fact the ATPase activity declines to about 30 % before much ATP is lost from the charcoal-treated sample. This must mean that the ATP remains quite tightly bound to the species arising during cold inactivation, but that it is removed after $(NH_4)_2SO_4$ precipitation of the protein.

Identification of nucleotide-binding forms of ATPase

Penefsky and Warner [4] have shown that during cold inactivation F_1 (11.9 S) is transformed into 9.1-S and 3.5-S species, the transformation of the protein to the

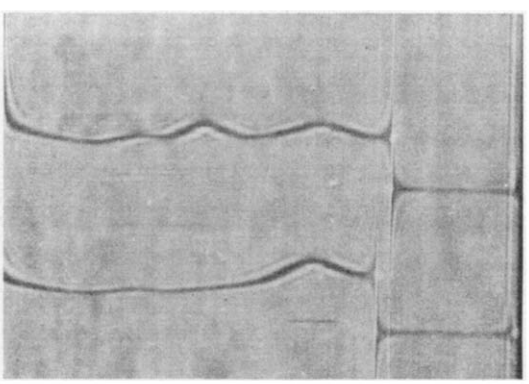


Fig. 8. The effect of $(NH_4)_2SO_4$ precipitation of cold-inactivated ATPase on its sedimentation behaviour. Washed ATPase was inactivated to 15 % of the initial activity by incubation at 0 °C in a buffer containing 10 mM Tris maleate, 150 mM KCl and 2 mM EDTA at pH 7.5. Half of the sample was precipitated with $(NH_4)_2SO_4$ and after spinning down dissolved in the same buffer. Since this sample contains about 200 mM $(NH_4)_2SO_4$ this was also added to the control sample. Both samples were examined in the ultracentrifuge. In each pattern sedimentation is from left to right. The phase angle was 30 °. The temperature of the rotor was 5 °C. Upper. Cold-inactivated ATPase. *s*: 11.3, 8.9, 3.1 S. Lower. Cold-inactivated ATPase precipitated with $(NH_4)_2SO_4$. *s*: 10.8 and 3.1 S. The Schlieren photographs shown are taken at a rotor speed of 55040 rev./min.

latter species being promoted by high concentrations of salt. A rational explanation of the experiments described above is that the active ATPase (11.9 S) contains bound nucleotides stable to $(NH_4)_2SO_4$ precipitation, and that at 0 °C this changes into the 9-S form, also containing bound nucleotides, which subsequently decomposes into the 3.5-S form which does not bind the nucleotides. Treatment of the 9-S form with high salt concentrations, for example by precipitating the enzyme with 50 % $(NH_4)_2SO_4$, increases its dissociation to the 3.5-S form and consequently, the loss of bound nucleotides to the solution. The experiment described in Fig. 8 supports this hypothesis. The ATPase was inactivated by about 85 % by incubation at 0 °C and two samples were taken. In one of these the enzyme was precipitated with an equal volume of saturated $(NH_4)_2SO_4$ and the enzyme redissolved in buffer. To the other a small amount of $(NH_4)_2SO_4$ was added to bring the final concentration of $(NH_4)_2SO_4$ to approximately the same value as that in the first sample after precipitating and redissolving (determined using Nessler's reagent). This was necessary since the presence of $(NH_4)_2SO_4$ in the solution was found to decrease the sedimentation coefficient by about 10 %. It is clear that the central peak (corresponding to the 9.1-S form) is missing from the precipitated sample, i.e. $(NH_4)_2SO_4$ precipitation,

TABLE II

INCORPORATION OF RADIOACTIVE NUCLEOTIDES DURING REACTIVATION OF COLD-INACTIVATED ATPase

Washed ATPase was inactivated at 0 °C in 1 ml of a buffer containing 10 mM Tris maleate and 50 mM KNO_3 at pH 7.5, at a protein concentration 5.4 mg/ml (Expt A), 2.7 mg/ml (Expt B) or 3.2 mg/ml (Expt C). Reactivation was started by addition of 2 ml of a 40 mM Tricine–NaOH, 37.5 % (w/v) glycerol buffer (pH 7.8) containing 3 mM [^{3}H]ATP (Expt A), 1.6 mM [^{3}H]ADP (Expt B) or 0.6 mM [^{3}H]GTP (Expt C). The reactivation was carried out at 30 °C. At intervals samples were taken from which the protein was freed from ambient nucleotides by repeated $(NH_4)_2SO_4$ precipitation. The final precipitate was clarified by centrifugation and the ATPase activity was determined. The amount of bound labelled nucleotide was determined in an $HClO_4$ extract of the protein.

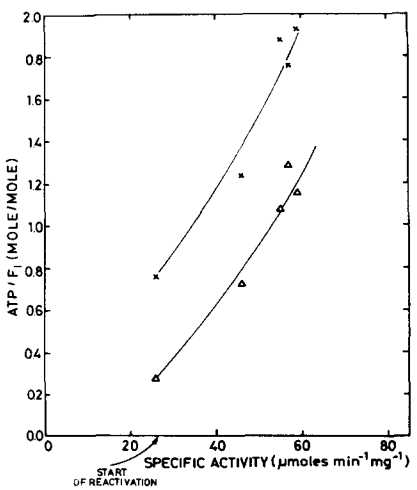
Expt	Reactivation time (min)	Specific activity (μmoles P_i $min^{-1} \cdot mg \cdot protein^{-1}$)	^{3}H-labelled nucleotide (mole/mole ATPase)
A	0	24	0.40
	3	34	0.81
	10	43	1.16
	30	46	1.26
	60	44	1.34
	120	49	1.56
B	0	38	0.40
	3	43	0.63
	10	43	0.73
	20	42	0.65
	50	43	0.85
	90	41	0.98
C	0	40	0.01
	3	58	0.06
	30	73	0.12
	78	79	0.25

as postulated, leads to a disappearance of the 9-S component and this is correlated with a loss of nucleotides from the protein to the solution.

Exchange of nucleotides on the 9-S form

As already reported, there is little exchange of nucleotides bound to the fully active ATPase with added nucleotides at room temperature. After 2 h only 0.8 mole of labelled ATP and 0.4 mole labelled ADP are exchanged [6]. The experiment described in Table II shows that the exchange is considerably stimulated during a cold inactivation–reactivation experiment. Up to 1.6 moles of labelled ATP can be incorporated per mole enzyme over a period of 2 h (Table II, Expt A). In a similar experiment (Fig. 9) total ATP and [^{3}H]ATP were measured during reactivation. These are here plotted against activity during reactivation rather than time. The lines corresponding to total and labelled ATP incorporated are approximately parallel. This suggests that all the "newly incorporated" ATP during reactivation comes from the solution, i.e. that the ATP on the 9-S form is freely exchangeable as is compatible with its weaker binding and that ATP bound to the 12-S form is not freely exchangeable.

Table II, Expt B, shows that the exchange of ADP is also stimulated, up to 1 mole labelled ADP being incorporated as compared to 0.4 mole per mole ATPase found with the native enzyme. As can be seen from Fig. 3B, there is little change in activity during reactivation in the presence of ADP. Fig. 10 shows also that there is less change in the ATP content of the enzyme under these conditions compared with the conditions of Fig. 9. Thus reactivation in the presence of ADP



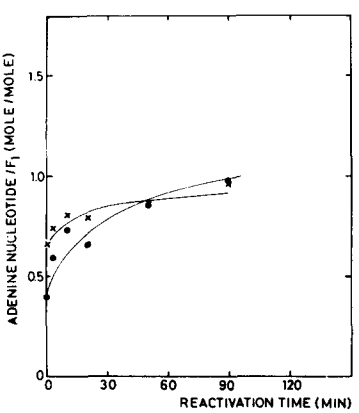


Fig. 9. ATP bound to ATPase during reactivation in the presence of ATP. Washed ATPase was inactivated at 0 °C, and reactivated as described in the legend to Table II, Expt A. Total ATP (×) and [^{3}H]ATP (△) bound to the washed enzyme was determined on $HClO_4$ extracts of the protein taken at various times during the reactivation.

Fig. 10. ATP bound to ATPase during reactivation in the presence of ADP. Washed ATPase was inactivated at 0 °C, and reactivated as described in the legend to Table II, Expt C. Total ATP (×) and [^{3}H]ADP (●) bound to the washed enzyme were determined on $HClO_4$ extracts of the protein taken at various times during the reactivation.

appears to lead to a decrease in the ATP/ADP ratio of the enzyme (the normal ratio of 1.5 falling to less than 1) and a simultaneous loss of activity.

Table II, Expt C, shows that, despite a considerable increase of activity when GTP is present during reactivation (see Fig. 3A), the incorporation of [^{3}H]GTP into the enzyme during renaturation is relatively small suggesting that this nucleotide is not freely exchangeable into the tight-binding sites on the 9-S unit. This was confirmed by ATP estimations on the enzyme during the experiment. Even in the presence of GTP the bound ATP increased, from 0.5 mole per mole enzyme with a specific activity of 28 to 1.5 moles per mole with a specific activity of 50, an increase similar to that obtained in the absence of GTP (see Fig. 6).

Sedimentation patterns of reactivated ATPase

In agreement with Penefsky and Warner [4], the reactivated enzyme gave a

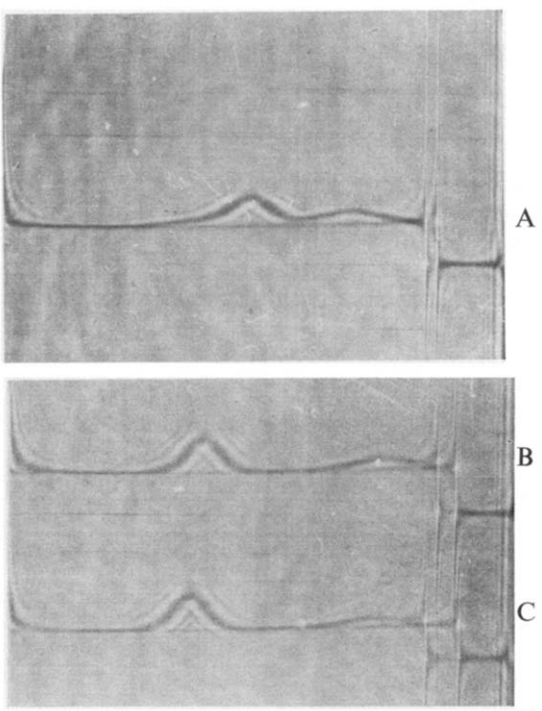


Fig. 11. Sedimentation behaviour of cold-inactivated ATPase and ATPase reactivated in the presence of ATP and ADP. Washed ATPase was inactivated at 0 °C in a buffer containing 3 mM Tris, 150 mM KCl, 2 mM EDTA at pH 7.5, at a protein concentration of 8 mg/ml. When the activity reached 10 % of the initial activity a sample was examined in the ultracentrifuge at 5 °C (A) and two samples were taken to which 4 mM ATP (B) or ADP (C) were added. These samples were brought to room temperature. The sample incubated in the presence of ADP did not show any increase in ATPase activity while that incubated in the presence of ATP reached an activity equal to 35 % of the original activity. Both samples were examined in the ultracentrifuge. In each pattern sedimentation is from left to right. The phase angle was 30 ° and the temperature of the rotor 5 °C (Expt A) or 20 °C (Expts B and C). A. ATPase inactivated to 10 % of the initial activity. *s*: 8.8, 3.5 S. B, ATPase reactivated in the presence of ATP. *s*: 12.4, 2.6 S. C, ATPase reactivated in the presence of ADP. *s*: 12.4, 2.6 S. The Schlieren photographs were taken at a rotor speed of 55290 rev./min.

peak in the ultracentrifuge indistinguishable from the native enzyme, at 12.1 S. As shown in Fig. 11, the ultracentrifuge pattern of enzyme reactivated in the presence of ATP was identical to that renatured in the presence of ADP, despite the difference in activity. This was also the case with the enzyme reactivated in the presence of GTP (not shown). Clearly, the amount of the 12-S peak is not directly correlated to the ATPase activity. Since no peak corresponding to the 9-S form was ever found after warming the cold-inactivated enzyme, it is likely that this is the form that changes to the 12-S form on warming, especially since the 3-S peak does not vary much during reactivation (Fig. 11).

DISCUSSION

We have seen that the bound nucleotides of beef-heart mitochondrial ATPase are released during cold inactivation, followed by $(NH_4)_2SO_4$ precipitation. The release of bound ATP, measured in this way, parallels the decrease of ATPase activity during cold inactivation. The slower loss of ADP indicates that this may be bound more tightly to the protein than ATP. This release of bound adenine nucleotide is not a consequence of the transformation of the ATPase into the 9-S component, but is caused by the subsequent $(NH_4)_2SO_4$ precipitation (and high ionic strength in general [4]) which accelerates the transformation of the 9-S component into 3-S subunits, which occurs only slowly during cold inactivation. After dissociation into the 3-S form (and loss of nucleotides) no reactivation can occur.

These findings can be accomodated in the following scheme.

$$\underset{(12.1\ S)}{F_1-Adn} \xrightarrow[(1)]{0\ ^\circ C} \underset{(9\ S)}{9\text{-S form}-Adn} \xrightarrow[(2)]{\text{irreversible}} \underset{(3.5\ S)}{Subunits+Adn}$$

$$\underset{30\ ^\circ C}{\longleftarrow}$$

Active ATPase, identified as the 12-S form of Penefsky and Warner [4], contains tightly bound nucleotides that are not readily exchangeable with the solution [6]. On incubation at 0 °C, this form changes into a 9-S form, which still contains nucleotides that are more readily exchangeable with nucleotides in the solution. The 9-S form has no ATPase activity. The 12-S and 9-S forms are in equilibrium, and conversion of the 9-S form to the 12-S form occurs when the protein solution is warmed. Non-specific aggregation is hindered by glycerol. It is uncertain as yet whether the 9-S form is lacking some of the subunits of the 12-S form or represents a different shape (less stream-lined) of the complete protein. Attempts are at present being made to isolate this species.

Since the 9-S species still contains the bound nucleotides, no additional nucleotides are necessary for transformation of this form into the 12-S unit. This transformation results in the bound nucleotides becoming stable again to $(NH_4)_2SO_4$ precipitation of the enzyme and the final 12-S unit is identical with the initial enzyme in ultracentrifuge pattern, activity and bound-ATP content. If ADP is present in solution, it is possible that this exchanges for some of the ATP from the tight sites. In this case, although the enzyme can reform an indistinguishable 12-S unit, the activity of the enzyme is not regained and the ATP/ADP ratio falls. It appears then that for full ATPase activity, the protein must have the correct ATP/ADP ratio of 1.5 and be

in the 12-S form.

The 9-S form, however, is of considerable theoretical interest. It has been postulated [18] that during ATP synthesis by the ATPase a conformational change in the ATPase is induced by electron transport, leading to increased dissociability and therefore exchangeability of the bound nucleotides. This postulated high-energy form of the enzyme bound to the membrane may be equivalent to the 9-S form in solution, the membrane preventing the 9 S → 3 S transition that can occur in solution. It is known that the membrane-bound ATPase is not cold labile. In this regard, it is interesting that the 9-S form has little, if any, ATPase activity, and also that it probably can exchange its ATP for ADP from the solution, unlike the 12-S form. ADP is considerably better than other diphosphates in this respect.

In solution at 0 °C, the 9-S form slowly dissociates irreversibly into the 3.5-S form, which very probably consists of separate subunits. Assuming a spherical shape, the 3.5-S units would have a molecular weight of about 50 000, about the size of the large subunits of ATPase (for a review see ref. 19). The 3.5-S unit contains no bound nucleotides. The transition of the 9-S form into the 3-S subunits is stimulated by high ionic strength [4], and in particular by $(NH_4)_2SO_4$ precipitation of the enzyme.

The stimulatory effect on the initial ATPase activity after prolonged incubation with GTP ($t_{\frac{1}{2}}$ 90 min) or PP_i (Table I) is probably caused by a slow binding of these compounds to the enzyme. The interaction of GTP is weak, as shown by the fact that no [^{3}H]GTP is found on the enzyme reactivated in the presence of [^{3}H]GTP. This explains why the increased ATPase activity declines to a normal value during the activity measurement.

Thus, there exist at least three distinguishable forms of beef-heart mitochondrial ATPase, differing in their sedimentation behaviour and in their interaction with nucleotides. Since the 12-S and 9-S units are interconvertible, it is tempting to postulate the involvement of both in the mechanism of oxidative phosphorylation previously outlined. What is clear, in any case, is that the correct amount of bound ATP is a necessary condition for ATPase activity. It is not, however, a sufficient condition, since the 9-S form contains bound ATP but is not active as an ATPase. Metastable forms of the ATPase containing bound GTP or PP_i also exist, and these have a higher ATPase activity than the normal enzyme. The slow release of these compounds from the protein, together with results suggesting two states of the active enzyme, differing in accessibility of –SH groups ([20], indicate unusual conformational properties of this enzyme that may be relevant to the mechanism of oxidative phosphorylation.

ACKNOWLEDGEMENTS

The authors are grateful to Miss Ineke van der Kraan for her expert technical assistance, and to Mr G. Dooijewaard and Mr Peter van Dijk for help with the ultracentrifuge experiments. This work was supported in part by grants from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O) under auspices of the Netherlands Foundation for Chemical Research (S.O.N.), and NATO research grant No. 563. D.A.H. is the recipient of a CIBA-Geigy European Fellowship.

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