BBA 75639

STEADY STATE KINETICS OF SOLUBLE AND MEMBRANE-BOUND MITOCHONDRIAL ATPASE

GORDON G. HAMMES AND DAVID A. HILBORN
Department of Chemistry, Cornell University, Ithaca, N.Y. 14850 (U.S.A.)
(Received December 14th, 1970)

SUMMARY

Steady state kinetic measurements of the rate of hydrolysis of ATP to ADP and inorganic phosphate by beef heart mitochondrial ATPase have been performed with both the solubilized enzyme and with the enzyme attached to a mitochondrial membrane fraction at 25° in 0.1 M NaCl with Mg^{2+} as the metal ion activator. These studies indicate the ATP Michaelis constants are somewhat larger for the soluble enzyme and the turnover numbers are considerably larger. In addition, the steady state parameters are essentially independent of pH over the range 7–9 for the membrane-bound enzyme, while the turnover number for the soluble enzyme varies considerably with pH. The product, ADP, is a competitive inhibitor of ATP and inhibits the soluble enzyme much more strongly than the membrane-bound enzyme. Oligomycin inhibits the membrane-bound enzyme very strongly, but has no effect on the activity of the soluble enzyme. The oligomycin inhibition is noncompetitive in nature.

INTRODUCTION

The mechanisms of action of soluble enzymes have been studied extensively for many years. Although it has been recognized that enzymes *in vivo* are often associated with membranes and that the properties of membrane-bound enzymes may be considerably different than their soluble counterparts, relatively few quantitative studies of this problem have been reported. Several approaches are possible. One method is to prepare the enzyme attached to the native membrane and compare it with a solubilized enzyme extracted from the membrane. In another method, soluble enzymes have been attached to solid matrices, which act as model membranes. Both of these approaches suffer from the drawback that the properties of the enzyme may be irreversibly altered by its extraction from the membrane or its attachment to the membrane.

A more satisfactory method is to separate the enzyme and membrane, and to reconstitute the original system by combining the solubilized enzyme with its native membrane. If the reconstituted system has the properties of the original membrane-bound enzyme, then it is reasonable to assume the structure of the enzyme has remained essentially intact throughout the preparative procedures. The ATPase of beef heart mitochondria is an example of this latter type of system. The solubilized

enzyme has been prepared in a quite pure form, and its physical and chemical properties have been studied extensively[1-4]. In addition, beef heart mitochondrial membrane fragments (ASU particles) have been prepared[4] which are devoid of ATPase activity but which readily associate with the soluble enzyme. The "reconstituted" enzyme has a number of properties similar to those of the original membrane-bound enzyme, but which are strikingly different from those of the soluble enzyme.

In this study, the pH-stat technique was used to determine the initial velocities of both the soluble and reconstituted membrane-bound ATPase activities over a range of ATP, ADP, oligomycin, and H^+ concentrations with Mg^{2+} as the metal ion activator. Differences between the steady state kinetic parameters of the soluble and membrane-bound enzyme have been observed, and the implications of these findings are discussed.

EXPERIMENTAL PROCEDURES

Reagents

ATP was obtained as the disodium salt from P-L Biochemicals, Inc.. Paper chromatography with the solvent system 0.15 M citric acid (pH 4.0)–95 % absolute ethanol–*n*-butanol (6:10:1 by vol.) indicated it was free of ADP contamination. ADP and $NADP^+$ were also obtained from P-L Biochemicals, Inc.. Oligomycin was purchased from Sigma Chemical Co., hexokinase from Calbiochem, and glucose-6-phosphate dehydrogenase from Boehringer Mannheim Corporation. All other chemicals used were analytical reagent grade.

Preparations

Two different methods of preparation have been used for the ATPase[4]. One of these methods yields an enzyme which contains an endogenous inhibitor. However, under the experimental conditions employed, the steady state parameters at pH 8.0 were identical, within experimental uncertainties, for the enzyme obtained with both methods. The preparation and characterization of ASU particles was as described elsewhere[4,5].

The solubilized ATPase was combined with the ASU particles using the following procedure. A suitable aliquot of ATPase of known activity in 0.025 M sucrose. 0.001 M EDTA, 0.01 M Tris buffer (pH 8.4) and an aliquot of a suspension of ASU particles (stored at −70°) were mixed together. The Mg^{2+} concentration was brought to $8 \cdot 10^{-4}$ M by adding $8 \cdot 10^{-2}$ M $MgSO_4$ to enhance binding of the enzyme to the particles[6]. After being incubated for 1 h at room temperature with intermittent stirring, the suspension was centrifuged at 12000 rev./min in a Sorvall SS-34 rotor for 10 min at room temperature. The supernatant was withdrawn carefully and its volume and activity measured. The precipitate was resuspended in 0.5 ml of a wash solution of the above buffer containing $8 \cdot 10^{-4}$ M Mg^{2+} and recentrifuged as above. The wash was also withdrawn carefully and its volume and activity measured. The membrane-bound enzyme was then suspended in the buffer containing $8 \cdot 10^{-4}$ M Mg^{2+} to the desired concentration (typically 600 μg ATPase/ml). The suspension was maintained at room temperature throughout the course of an experiment and was never used more than 8 h after its preparation. No change in activity was observed during this time period.

Analytical methods

Protein concentration for the solubilized ATPase was determined using the method of LOWRY *et al.*[7] with human serum albumin as the standard. The result was divided by 1.18 to obtain the dry weight of the enzyme[8]. The biuret method in the presence of 0.33 % deoxycholate was used to determine the concentration of ASU particles[9].

The amount of ATPase bound to the ASU particles was determined by subtracting the activity found in the supernatant and wash solutions from the original amount added. An excess of the ATPase was generally used to insure saturation of the ASU particles with ATPase; however, in cases where an excess of particles was present up to 90 % of the original ATPase was absent from the supernatant. The ATPase appeared to be very tightly bound to the ASU particles since less than 1 % of the original enzymatic activity was found in the wash solutions.

Adenylate kinase activity of the ASU particles was assayed using a modification of the method described by RHOADS AND LOWENSTEIN[10]. The formation of ATP was coupled to the glucose-6-phosphate dehydrogenase reaction with hexokinase. The glucose-6-phosphate dehydrogenase reaction was followed by observing the increase with time of the absorbance at 340 nm corresponding to NADPH formation. The experimental conditions were 50 mM glucose, 0.62 mM $NADP^+$, $5 \cdot 10^{-4}$ M $MgCl_2$; the concentrations of hexokinase and glucose 6-phosphate were such that the rate of the coupled reaction was about 0.5 μmoles NADPH formed per min per ml reaction volume when the ATP concentration was 0.6 mM. 4 μg of oligomycin per ml of reaction volume were added to inhibit any endogenous ATPase activity which would have caused the adenylate kinase activity to be underestimated (Y. KAGAWA, personal communication). The ADP concentration was $5 \cdot 10^{-4}$ M.

Initial velocity measurements of the ATPase reaction were made using the pH-stat technique with the Radiometer Type TTTlb Titrator and Type TTTA31 microtitration assembly.

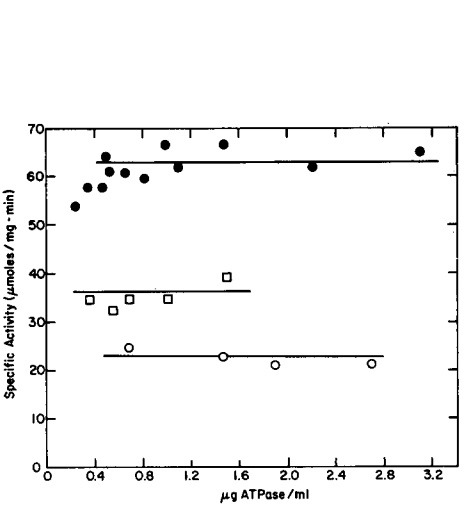
For all experiments except ADP inhibition experiments the reaction mixtures contained 0.1 M NaCl, $5 \cdot 10^{-3}$ M $MgSO_4$, and various concentrations of ATP and oligomycin in a final volume of 4 ml. Because the inhibition of the enzyme by ADP was observed to vary with the amount of Mg^{2+} added to the reaction mixture, the total concentration of Mg^{2+} was varied during the ADP inhibition experiments so that both the uncomplexed Mg^{2+} and the percent ADP complexed with Mg^{2+} were kept constant. The free Mg^{2+} was maintained at $2.0 \cdot 10^{-3}$ M so that 74 % of the ADP added was complexed with the metal[11]. For activity assays the ATP concentration was $2.5 \cdot 10^{-3}$ M. The ATPase, stored at 4° as the 50 % $(NH_4)_2SO_4$ precipitate, was dissolved in buffer (0.025 M sucrose, 0.001 M EDTA, 0.01 M Tris, pH 8.4) to the desired concentration, and 1 μmole ATP was added per ml solution to stabilize the enzyme[1]. The solution stood at least 1 h before use and its activity was not observed to change for several days (although kinetic studies were always performed within 24 h). A 15–30-μl aliquot of the ATPase was introduced into the reaction volume to initiate the reaction. Initial velocities were obtained from the resulting graphs of base added *versus* time. A period of about 15 sec after introduction of the enzyme was neglected due to a readjustment of the pH of the reaction mixture. Initial velocities were corrected for a very small change in pH due to CO_2 absorption (usually less than 1 % of the measured rate), and for the fact that less than one mole of H^+ is produced per

mole of ATP hydrolyzed at some of the pH values studied[12]. Initial velocities were determined for the reconstituted enzyme as in the case of the soluble ATPase except that a small additional correction (less than 2 % of the measured rate) was made for endogenous ATPase activity of the particles alone. Some rate measurements were also made by analyzing for inorganic phosphate in the presence of an ATP regenerating system previously described[1] for comparison with the results obtained with the pH-stat assay.

The initial rate data were analyzed using a weighted least squares analysis of double reciprocal plots, while an unweighted least squares analysis was used on secondary plots associated with inhibition by oligomycin. Experimental errors were estimated to be ± 15 % for all kinetic parameters except for oligomycin inhibition constants where the error is ± 20 %.

RESULTS

The specific activity of the ATPase was measured as a function of enzyme concentration (Fig. 1). For the range 0.4–3.1 μg/ml the specific activity of the solubilized enzyme was independent of enzyme concentration, but below values of 0.4 μg/ml the specific activity appeared to decrease. All experiments with the solubilized ATPase were performed at enzyme concentrations above this value. The specific activity of the membrane-bound ATPase (based on the amount of ATPase, not on the total membrane protein) also showed no dependence on enzyme concentration over the concentration range utilized in these experiments (Fig. 1). The enzyme concentration used in both cases was approx. 1–2 μg/ml.



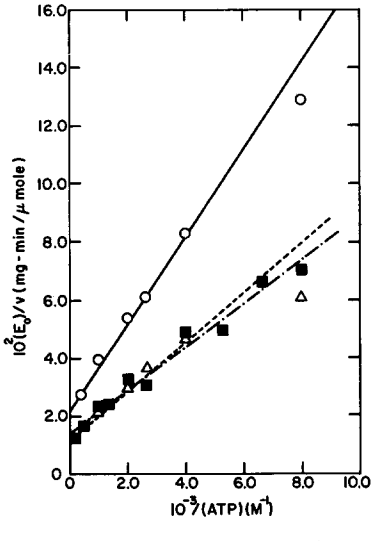


Fig. 1. Specific activity of soluble and membrane-bound ATPase as a function of protein concentration. Activities were assayed as described in the text. The data are for membrane-bound ATPase, pH 7.0 (○); membrane-bound ATPase, pH 8.0 (□); solubilized ATPase, pH 8.0 (●).

Fig. 2. Double reciprocal plots of initial rate data for the soluble ATPase. ○—○, pH 7.0; ■---■, pH 8.0; △–·–△, pH 9.0. The lines represent a weighted least squares analysis of the data. Experimental conditions are given in the text.

Michaelis constants (K_s) and turnover numbers (k_s) were determined at pH 7.0, 8.0, and 9.0 for the solubilized enzyme. The range of ATP concentrations was $2.50 \cdot 10^{-3}$ $-1.25 \cdot 10^{-4}$ M. The enzyme obeyed Michaelis–Menten kinetics within experimental error as shown by the double reciprocal plots in Fig. 2. The resulting steady state parameters are listed in Table I. Addition of oligomycin up to a concentration of $1 \cdot 10^{-6}$ M had no discernible effect on the initial velocities.

Double reciprocal plots of the initial velocity data for the membrane-bound

TABLE I

KINETIC PARAMETERS FOR MITOCHONDRIAL ATPase

	Solubilized ATPase			*Membrane-bound ATPase*		
pH:	*7.0*	*8.0*	*9.0*	*7.0*	*8.0*	*9.0*
$10^6 \times k_S$ (mole/mg per min)	45.4	89.3	71.9	28.0	29.9	32.7
$10^4 \times K_S$ (M)	7.0	7.90	5.47	3.86	3.15	3.87
$10^2 \times (k_S/K_S)$ ($mg^{-1} \cdot min^{-1}$)	6.5	11.3	13.1	7.3	9.5	8.4
Oligomycin: K_I (μM)	—	—	—	0.15	0.91	0.71
ADP: K_I (μM)	—	30	—	—	80	—

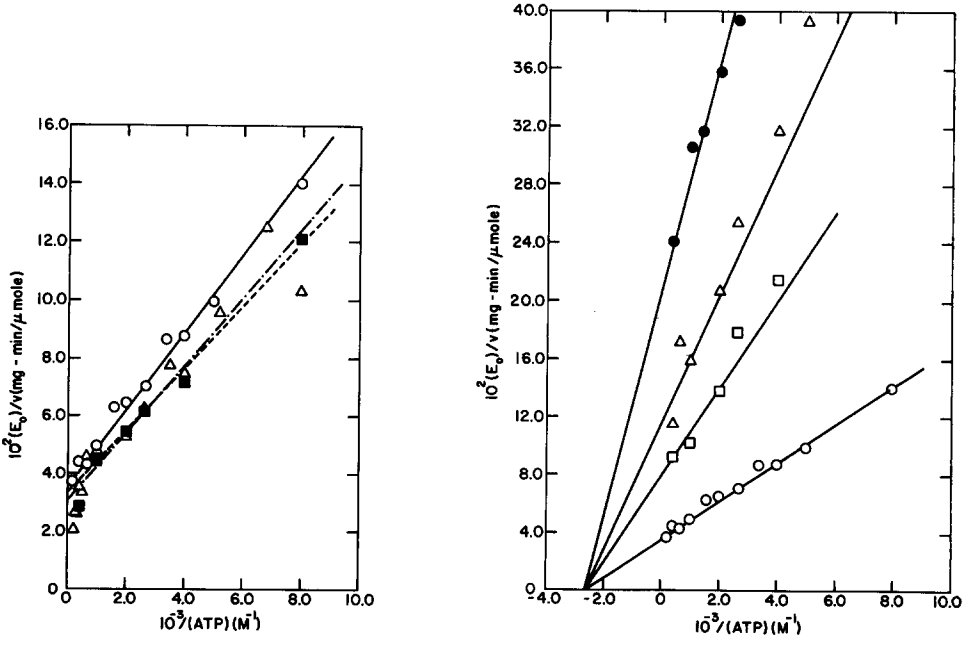


Fig. 3. Double reciprocal plots of initial rate data for the membrane-bound ATPase. ○—○, pH 7.0; ■---■, pH 8.0; △-·-△, pH 9.0. The lines represent a weighted least squares analysis of the data. Experimental conditions are given in the text.

Fig. 4. Double reciprocal plots of oligomycin inhibition data for the membrane-bound ATPase at pH 7.0. The plots are of the reciprocal initial rates *versus* 1/[ATP] at constant oligomycin concentrations: ○, no oligomycin; □, $9.0 \cdot 10^{-8}$ M; △, $3.0 \cdot 10^{-7}$ M; ●, $6 \cdot 10^{-7}$ M. The lines represent a weighted least squares analysis of the data assuming a common intercept on the abscissa. Experimental conditions are given in the text.

ATPase are shown in Fig. 3. Small deviations from Michaelis–Menten kinetics, in a manner expected for substrate activation, were observed at high substrate concentrations at pH 8.0 and 9.0. However, the ATP concentration was comparable to the Mg^{2+} concentration in these cases, so that uncomplexed ATP may account for the activation. Since the double reciprocal plots for the membrane-bound ATPase were linear at low substrate concentrations, the weighted least square lines shown in Fig. 3 were calculated neglecting substrate concentrations greater than $2.5 \cdot 10^{-3}$ M. The values of the Michaelis constants and turnover numbers are included in Table I.

Figs. 4–6 show the result of inhibition of the membrane bound ATPase by oligomycin at pH 7.0, 8.0 and 9.0, respectively. In all cases the oligomycin concentration was at least 10 times that of the enzyme. A simple mechanism consistent with this data is that predicted for noncompetitive inhibition. The mechanism can be formulated most simply as

$$\begin{aligned} &E + \text{ATP} \overset{K_S}{\rightleftharpoons} X \rightarrow E + P_i + \text{ADP} \\ &E + I \overset{K_I}{\rightleftharpoons} EI \\ &X + I \overset{K_I}{\rightleftharpoons} XI \end{aligned} \qquad (1)$$

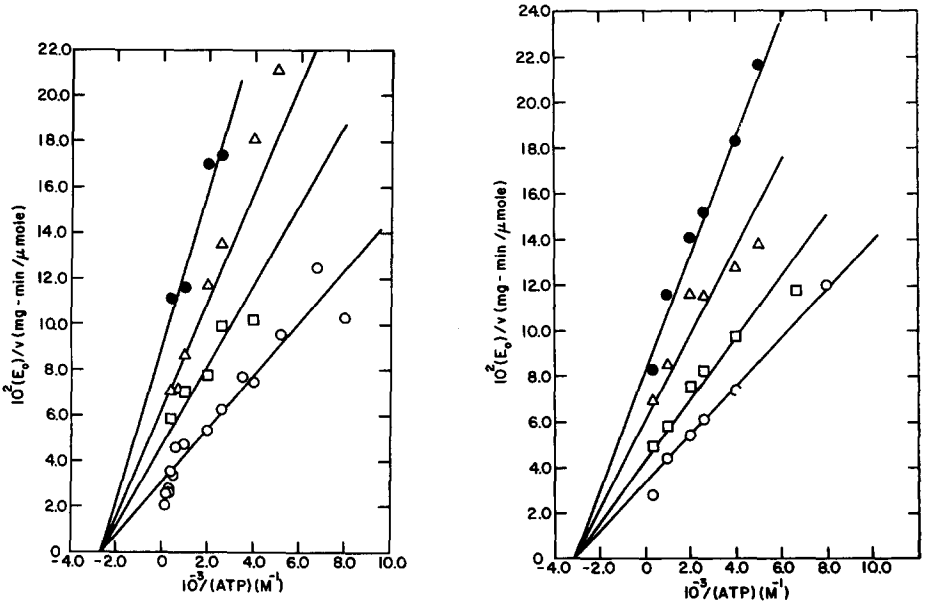


Fig. 5. Double reciprocal plots of oligomycin inhibition data for the membrane-bound ATPase at pH 8.0. The plots are the reciprocal initial rates *versus* 1/[ATP] at constant oligomycin concentrations: ○, no oligomycin; □, $9 \cdot 10^{-8}$ M; △, $3 \cdot 10^{-7}$ M; ●, $1 \cdot 10^{-6}$ M. The lines represent a weighted least squares analysis of the data assuming a common intercept on the abscissa. Experimental conditions are given in the text.

Fig. 6. Double reciprocal plots of oligomycin data for membrane-bound ATPase at pH 9.0. The plots are reciprocal initial rates *versus* 1/[ATP] at constant oligomycin concentrations: ○, no oligomycin; □, $5 \cdot 10^{-8}$ M; △, $6 \cdot 10^{-7}$ M; ●, $1 \cdot 10^{-6}$ M. The lines represent a weighted least squares analysis of the data assuming a common intercept on the abscissa. Experimental conditions are given in the text.

where E is the enzyme–membrane complex, X is a reaction intermediate, I is oligomycin, K_S is the Michaelis constant for ATP and K_I is the inhibitor dissociation constant. The reciprocal initial velocity can be written as

$$\frac{1}{v} = \frac{1}{V_S}\left[1 + \frac{(I)}{K_I}\right] + \frac{K_S}{[\mathrm{ATP}]V_S}\left[1 + \frac{(I)}{K_I}\right] \qquad (2)$$

If, as assumed, the oligomycin binding constants for both E and X are identical the plots of $1/v$ *versus* $1/[\mathrm{ATP}]$ shown in Figs. 4–6 should intersect at a common point on the $1/[\mathrm{ATP}]$ axis. The lines resulting from a weighted least squares analysis of the data intersect at a common point well within the experimental uncertainty thus confirming the mechanism of Eqn. 1. Simple uncompetitive and competitive inhibition schemes are inconsistent with the data. The value of the intercept at each pH was found by assuming various values for the point of intersection and determining which value minimized the standard deviation of a weighted least squares analysis of the data. The final lines resulting from this procedure are shown in Figs. 4–6. Secondary plots of the intercepts and slopes *versus* oligomycin concentration were then constructed to determine the inhibition constants. The constants obtained are given in Table I.

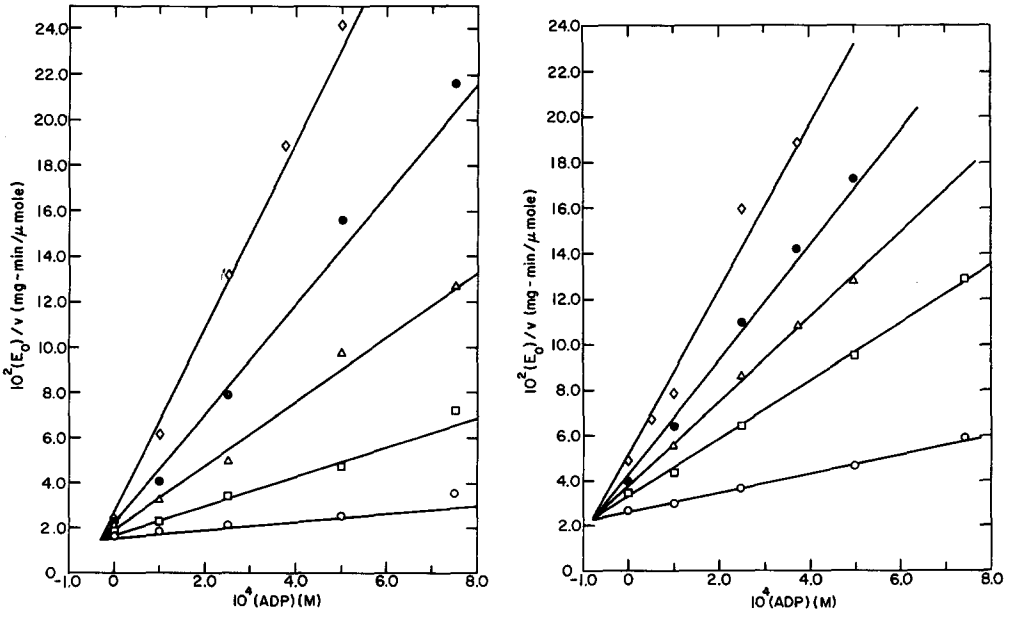


Fig. 7. ADP inhibition of the solubilized ATPase, pH 8.0. The data are plotted as reciprocal initial velocities *versus* ADP concentrations at fixed initial concentrations of ATP: ○, $2.6 \cdot 10^{-3}$ M; □, $1.0 \cdot 10^{-3}$ M; △, $0.51 \cdot 10^{-3}$ M; ●, $0.38 \cdot 10^{-3}$ M; ◇, $0.26 \cdot 10^{-3}$ M. The lines represent a weighted least squares analysis of the data assuming pure competitive inhibition, *i.e.*, assuming a common intercept. Experimental conditions are given in the text.

Fig. 8. ADP inhibition of the membrane-bound ATPase, pH 8.0. The data are plotted as reciprocal initial velocities *versus* ADP concentrations at fixed initial concentrations of ATP: ○, $2.6 \cdot 10^{-3}$ M; □, $1.0 \cdot 10^{-3}$ M; △, $0.66 \cdot 10^{-3}$ M; ●, $0.51 \cdot 10^{-3}$ M; ◇, $0.38 \cdot 10^{-3}$ M. The lines represent a weighted least squares analysis of the data assuming pure competitive inhibition, *i.e.*, assuming a common intercept. Experimental conditions are given in the text.

The inhibition of the ATPase reaction by ADP was investigated at pH 8.0. The results are summarized in Figs. 7 and 8 in plots of reciprocal velocities at constant ATP concentrations against inhibitor (ADP) concentrations. A competitive inhibition mechanism predicts that the lines at constant ATP concentration intersect at a common point and that the value of this point on the abscissa is the negative of the inhibition constant. This mechanism is consistent with the data. Using a procedure similar to that described above, the values of the inhibition constants were determined by assuming a common intersection point. The final lines obtained are shown in Figs. 7 and 8, and the ADP inhibition constants are given in Table I.

The adenylate kinase activity of the ASU particles was assayed in order to be certain decomposition of ADP was not appreciable during ADP inhibition experiments. The activity was found to be 20 nmoles ATP produced per mg protein per min at a concentration of $5 \cdot 10^{-4}$ M ADP. The lowest ADP concentration in the ADP inhibition studies was $5 \cdot 10^{-5}$ M ADP (or a total of 200 nmoles ADP per reaction volume) with a maximum of 0.2 mg ASU particles. Since the initial velocity was taken after 15 sec, less than 1 % of the ADP was decomposed. This indicates that the adenylate kinase activity can be neglected.

Experiments utilizing an ATP regenerating system in ATPase assays have suggested that the activity of the enzyme is not affected by being associated with its membrane[13]. Since the results presented here indicate that the turnover number of the ATPase is decreased when it becomes membrane bound, experiments were carried out with the ATP regenerating system to reconcile the difference. The time course of the soluble enzymatic reaction obtained using the ATP regenerating system is shown in Fig. 9 and agrees well with published results[1]. The time course of the reaction for the membrane-bound enzyme is also shown in Fig. 9. With this information the discrepancy can be explained. The pH-stat method measures initial velocities, while the ATP regenerating system is used for a specified time period, usually 10 min[1]. Because

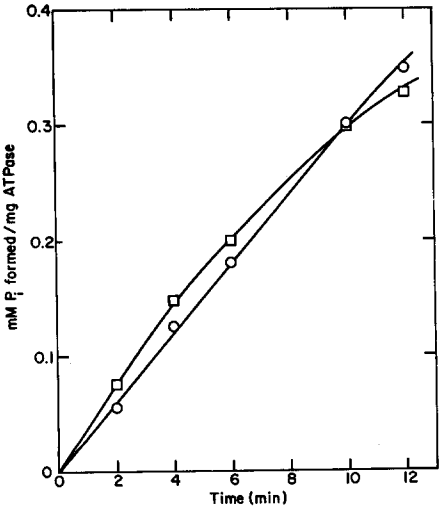


Fig. 9. Rate of ATP hydrolysis determined by analyzing for inorganic phosphate in the presence of the ATP regenerating system[1] with (○—○) membrane-bound ATPase and (□--□) solubilized ATPase. The MgATP concentration was $5 \cdot 10^{-3}$ M.

of the nonlinear time course for the soluble enzyme the ATP regenerating system yields a lower velocity than an initial velocity measurement would. In fact, the initial velocity obtained with the regenerating system is identical, within experimental error, with that obtained with the pH stat assay. On the other hand, the pH-stat method and the 10-min point of the ATP regenerating system assay should yield identical results with the membrane bound enzyme since the time course is quite linear; this is observed.

DISCUSSION

The steady state kinetic parameters are clearly different for the soluble and membrane-bound enzyme. The Michaelis constant is consistently larger for the soluble enzyme, although the difference is not great. The turnover number of the soluble enzyme is also greater than that of the membrane-bound enzyme with the maximum difference being a factor of three at pH 8.0. The Michaelis constants for beef heart mitochondria are similar in magnitude to those found for other ATPases[14–16], although this parameter has been obtained under a wide variety of conditions.

A comparison between bound and soluble enzymes has been made in other systems, but few have actually investigated kinetic parameters. SONÉ *et al.*[15] have found that both the membrane-bound and soluble ATPase from baker's yeast have the same Michaelis constant for ATP but that their maximum velocities differ, with that of the membrane-bound enzyme being lower. However, a reconstituted system was not used in their study. Soluble enzymes have been attached to solid matrices[17]; these enzymes have been found to undergo a variety of changes in their kinetic parameters[18]. These changes are generally highly dependent on the charge of both substrate and matrix. In a study of membrane-bound and soluble acetylcholine esterase using the pH-stat technique[19], the lower reaction velocity of membrane-bound acetylcholine esterase was attributed to a localized increase in H^+ concentration in an unstirred region around the membrane. This, coupled with a decrease in the intrinsic activity with decreasing pH, caused the lowered velocity. Addition of a buffer to the reaction mixture increased the activity to that of the solubilized enzyme. This explanation, however, probably does not account for the decrease in activity of the beef heart mitochondrial ATPase when it becomes bound to the membrane since both the pH stat and the buffered ATP regenerating system give the same velocities with the reconstituted system.

A difference in activities between the soluble and membrane-bound ATPase from beef heart mitochondria was observed previously, but the difference was attributed to a naturally occurring ATPase inhibitor[4]. In the present study, a high salt concentration was used which virtually abolishes the activity of the inhibitor[4]. To further test the effect of the inhibitor, an inhibitor-free ATPase preparation was reconstituted with inhibitor-free ASU particles. A difference in activities between the soluble and membrane-bound ATPase was again observed.

The Michaelis–Menten parameters are essentially independent of pH for the membrane-bound enzyme, but the turnover number of the soluble enzyme changes with pH. The values of k_S/K_S are included in Table I; the pH dependence of this quantity is determined by the ionization properties of the free enzyme and suggests an ionizable group in the enzyme with a pK of about 7 is essential for activity.

The effect of this group is apparently masked in the membrane-bound enzyme, possibly due to a structural alteration or conformational change.

A possible cause of the general lowering of velocities in the reconstituted system may be steric hindrance of the enzyme–substrate interactions by the membrane. However, this simple explanation does not account for the different relative rates at each pH. Another factor to consider is charge–charge interactions. At the pH values studied the ATP or MgATP complex is negatively charged. If the membrane surface has a net negative charge, repulsion of the substrate might cause the decrease in activity. However, a comparison of the Michaelis constants indicates that the substrate interacts more strongly with the membrane-bound enzyme which is contrary to the expectation for this model. Furthermore, repulsive interactions should be weaker at pH 7 than at pH 9; however, no appreciable difference in K_S is observed.

The product ADP binds more tightly to the soluble enzyme than to the membrane-bound enzyme. The difference in the inhibition constants is qualitatively reflected in both the pH stat and the ATP regenerating system assays. For both assays, the rate was considerably more linear with time for the reconstituted system than for the solubilized ATPase.

The pH dependence of the oligomycin inhibition is difficult to interpret since it may be due to functional groups on either the membrane or the oligomycin or both. The noncompetitive nature of the inhibition on the membrane-bound enzyme, however, is consistent with the demonstration that oligomycin binds at a site on the membrane rather than the ATPase[8]. The mechanism of this type of inhibition implies that the inhibitor binds to both the enzyme–membrane system and the substrate–enzyme–membrane complex. A study of the oligomycin inhibition of Na^+–K^+-stimulated membrane-bound ATPase from beef brain has indicated a different mechanism is operative; in this case oligomycin is an uncompetitive inhibitor of the enzyme[20].

This study has emphasized some of the major differences in the steady state properties of soluble and membrane-bound ATPase. Work is now in progress to examine the elementary steps involved in the binding and catalytic reactions.

ACKNOWLEDGMENTS

We would like to thank Professor E. Racker for many helpful discussions and suggestions regarding this study and for providing us with beef heart mitochondria. This work was supported by a grant from the National Institutes of Health (GM 13292). D.A.H. was a National Institutes of Health Trainee (GM 00834).

REFERENCES

1 M. E. PULLMAN, H. S. PENEFSKY, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3322.
2 H. S. PENEFSKY AND R. C. WARNER, *J. Biol. Chem.*, 240 (1965) 4694.
3 H. S. PENEFSKY, *J. Biol. Chem.*, 242 (1967) 5789.
4 L. L. HORSTMAN AND E. RACKER, *J. Biol. Chem.*, 245 (1970) 1336.
5 J. M. FESSENDEN-RADEN, *J. Biol. Chem.*, 244 (1969) 6662.
6 B. BULOS AND E. RACKER, *J. Biol. Chem.*, 243 (1968) 3891.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 Y. KAGAWA AND E. RACKER, *J. Biol. Chem.*, 241 (1966) 2461.
9 E. E. JACOBS, M. JACOB, D. R. SANADI AND L. B. BRADLEY, *J. Biol. Chem.*, 223 (1956) 147.
10 D. G. RHOADS AND J. M. LOWENSTEIN, *J. Biol. Chem.*, 243 (1968) 3963.

11 E. WALAAS, *Acta Chem. Scand.*, 12 (1958) 528.
12 R. A. ALBERTY, *J. Biol. Chem.*, 243 (1968) 1337.
13 G. SCHATZ AND J. SALTZGABER, *Biochim. Biophys. Acta*, 180 (1969) 186.
14 M. J. SELWYN, *Biochem. J.*, 105 (1967) 279.
15 N. SONÉ, E. FURUYA AND B. HAGIHARA, *J. Biochem.*, 65 (1969) 935.
16 G. SCHATZ, H. S. PENEFSKY AND E. RACKER, *J. Biol. Chem.*, 242 (1967) 2552.
17 R. GOLDMAN, O. KEDEM, I. H. SILMAN, S. R. CAPLAN AND E. KATCHALSKI, *Biochemistry*, 7 (1968) 486.
18 E. M. CROOK, *Biochem. J.*, 107 (1968) 1P.
19 H. I. SILMAN AND A. KARLIN, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1664.
20 C. E. INTURRISI AND E. TITUS, *Mol. Pharmacol.*, 4 (1968) 591.