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Kinetic and Thermodynamic Properties of Beef Heart Mitochondrial ATPase: Effect of Co-Solvent Systems[1]

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Abstract

The effects of glycerol and methanol upon beef heart mitochondrial ATPase (F_1) were studied. Glycerol was found to be a potent reversible inhibitor of the F_1-catalyzed hydrolysis of ATP and ITP. The inhibition of ATP hydrolysis was linear with respect to glycerol concentrations, while that of ITP was not. From the temperature dependence of V_{max} for F_1-catalyzed ATP and ITP hydrolysis in glycerol or methanol solutions, the energy of activation and the enthalpy of activation were calculated. The inhibitory effect of ADP on F_1 hydrolytic activity was studied in three solvent systems (totally aqueous, 20% methanol, and 20% glycerol). Compared to the aqueous system, methanol decreased the potency of ADP as an inhibitor, and glycerol enhanced the potency.

Introduction

Efforts to gain an understanding of the regulation of mitochondrial ATPase (F_1) have uncovered numerous points of control. Investigation has brought to light a large number of activators and inhibitors of F_1-ATPase. Among these are substrate analogs [1–9] and organic solvents [10]. The existence of a regulatory as well as a catalytic site has been proposed as a model of F_1-ATPase activity in order to account for the observed phenomena [5, 9, 11, 12]. It was further postulated that adenine nucleotide binding to the regulatory site decreased the activity of the catalytic site. No information regarding the mechanism of operation of the proposed regulatory site has been found. The rate-limiting steps for either F_1-catalyzed ATP synthesis or hydrolysis

[1] Abbreviations used: TEA, triethanolamine; PEP, phosphoenolpyruvate; ATP, adenosine-5′-triphosphate; ITP, inosine-5′-triphosphate; ADP, adenosine-5′-diphosphate.

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have not yet been established, although a proposal has been made by Boyer and co-workers [13–16]. Analysis of the effects of organic solvents on kinetic and thermodynamic properties of an enzyme provides a tool for elucidation of the control and mechanism of an enzymatic reaction. Examples of earlier work with organic co-solvent systems on other enzymes have been provided by Gautney et al. [17], Tan and Lovrien [18], and Pesheck and Lovrien [19]. This paper presents studies of the effects of methanol and glycerol upon the rate of F_1-catalyzed ATP and ITP hydrolysis.

Materials and Methods

Beef heart mitochondrial ATPase (F_1) was prepared by the method of Knowles and Penefsky [20] or Spitsberg and Blair [21]. Specific activities for these preparations were 100 μmoles min^{-1} mg^{-1}. Initial velocity experiments were performed in a total volume of 1.0 ml at 20°C unless otherwise noted in the figures. The reaction buffer in the coupled enzyme assays consisted of 50 mM TEA-Cl, pH 8.0, 1 mM KCl, 0.8 mM PEP, 0.3 mM NADH, 0.02 mg pyruvate kinase, and 0.02 mg lactate dehydrogenase. Nucleotide and magnesium concentrations were saturating at all temperatures. Rate of reaction was determined by disappearance of NADH as measured by monitoring the change in absorbance at 340 nm on a GCA/MacPherson double-beam spectrophotometer. All rates measured were initial velocities and represent V_{max} under the conditions in which the experiment was carried out. The effect of organic solvents on the coupling enzymes was found to be negligible under the experimental conditions.

The data presented in Fig. 5 was obtained by monitoring the release of inorganic phosphate due to ATP hydrolysis. The reaction buffer for the phosphate release assays contained 50 mM TEA, pH 8.0, 1 mM KCl, 2.0 mM $MgCl_2$, 0.6 mM ATP, and ADP concentrations as noted in the figure legends. Reaction volume was 2.0 ml. After 30 min of reaction time, the reaction was stopped by addition of 0.20 ml 70% perchloric acid. Measurement of phosphate released was performed by the method of Peterson [22].

Reactions were initiated by the addition of F_1 that had previously been centrifuged at 3°C from a 60% saturating $(NH_4)_2SO_4$ suspension in order to remove $(NH_4)_2SO_4$ and nucleotides present in the storage buffer, then resuspended with 0.25 M sucrose. Concentrations of organic solvents, when used, have been noted in the figure legends.

Best-fit lines and thermodynamic quantities were calculated by a microcomputer (North Star Computers, Inc.) using standard statistical methods.

Results

Glycerol was found to be an inhibitor of F_1-catalyzed hydrolysis of ATP. It was slightly less potent in its inhibition of ITP hydrolysis (Fig. 1). Inhibition of ATP hydrolysis was found to be linear with glycerol concentration. Inhibition of ITP hydrolysis was found to be nonlinear. Significant inhibition of ITP hydrolysis did not appear until approximately 10% (v/v) of the reaction solution was glycerol. Inhibition of ATP hydrolysis occurred upon addition of much smaller volumes of glycerol. Inhibition was not time dependent when either nucleotide was used as the F_1 substrate. The inhibition was reversible for both nucleotides (data not shown). Despite the apparent similarities in the structures of ATP and ITP, their hydrolysis by F_1 appeared to be mediated or controlled differently.

Lineweaver–Burk plots for ATP and ITP hydrolysis are presented in Figs. 2a and 2b respectively. F_1-catalyzed ATP hydrolysis in neat solutions produces Lineweaver–Burk plots indicating negative cooperativity [5, 7, 9].

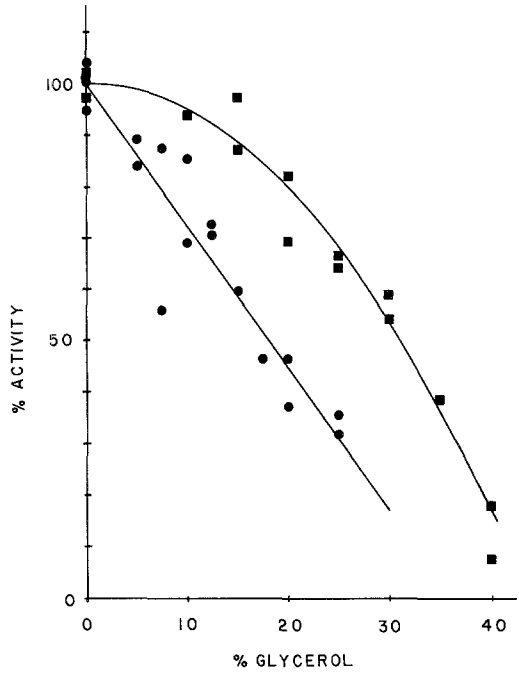

Fig. 1. ATP hydrolysis (●) and ITP hydrolysis (■) activity as a function of glycerol concentration.

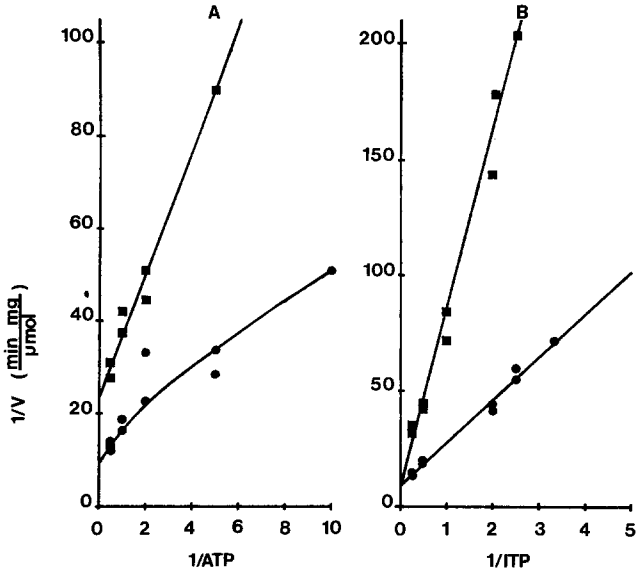

Fig. 2. Plot of 1/ATP concentration vs. 1/velocity in aqueous (●) and 23% glycerol buffer (■). Analogous results are obtained with different glycerol concentrations (data not shown).

Glycerol eliminates this curvature. It was previously demonstrated that many effectors, such as activating anions [7, 9] and 20% (v/v) methanol [10], eliminate the observed negative cooperativity. In Fig. 2b we see that double-reciprocal plots of ITP hydrolysis in neat and 33% (v/v) glycerol reaction media produce straight-line plots. These results also indicate that the control or mechanisms of F_1-catalyzed hydrolysis of ATP and ITP are different. Other investigations have shown that, in the presence of some anions (7) and organic solvents [10], the shape of double-reciprocal plots of ATP hydrolysis changed while that of ITP did not.

From transition state theory we know that

$$k = \frac{\kappa RT}{N_A h} e^{\Delta S^*/R} e^{-\Delta H^*/RT} \tag{1}$$

where k is the rate constant, κ is the transmission coefficient, R is the gas constant (8.314 J K^{-1} mol^{-1}), N_A is Avogadro's number, h is Planck's constant (6.626×10^{-34} J sec), T is the temperature in Kelvin, ΔS^* is the entropy of activation, and ΔH^* is the enthalpy of activation. Assuming $\kappa = 1$ and taking the logarithm of both sides, we have

$$\ln k = (\Delta S^*/R - \Delta H^*/RT) + \ln \frac{RT}{N_A h} \tag{2}$$

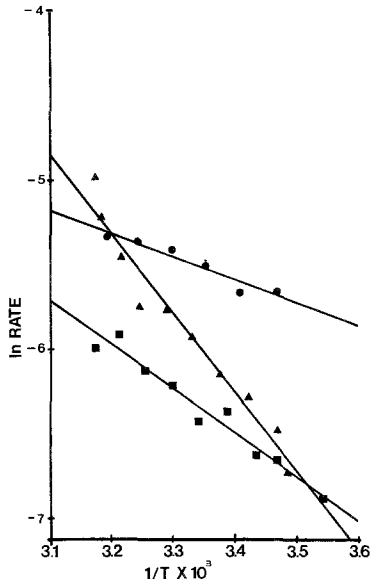

Fig. 3. Arrhenius plot of ATP hydrolysis in aqueous (●), 20% methanol (▲), and 20% glycerol (■) buffers. Rates were measured as change in absorbance per second. Temperatures were measured as degrees Kelvin.

Thus a plot of $1/T$ vs. the logarithm of observed rates at varying temperatures allows calculation of ΔH^* and E_a.

The necessary experiments were performed to determine these quantities for F_1-catalyzed hydrolysis of ATP and ITP in aqueous, 20% methanol, and 20% glycerol media. The activity of water, as determined by the pK of various acids and bases, was not significantly different from that of totally aqueous solutions [23]. The results of the experiments are presented in Figs. 3 and 4 and tabulated in Table 1.

The F_1-catalyzed hydrolysis of ATP has previously been shown to be stimulated by 20% (v/v) methanolic solution [10] when the assay temperature was 30°C. The previous results were obtained in the presence of 0.2 M sucrose, while the experiments described here were performed in the absence of sucrose. It is interesting that in the absence of sucrose, methanolic solutions only stimulate ATP hydrolysis at temperatures above 40°C (Fig. 3). As temperature decreases, methanolic solutions exhibit more potent inhibition of ATP hydrolysis. Under all the assay temperatures at which assays were performed, 20% (v/v) glycerol inhibited ATP hydrolysis (Fig. 3).

When F_1-catalyzed ITP hydrolysis was monitored at temperatures above about 17°C, methanolic solutions stimulated hydrolysis, and glycerol solutions inhibited it (Fig. 4). This should be contrasted with the results reported previously [10] for experimental conditions which contained 0.2 M sucrose.

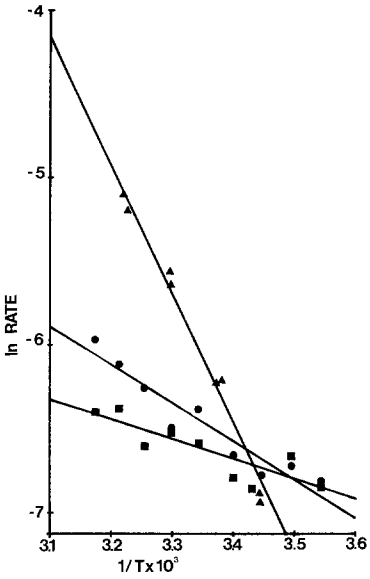

Fig. 4. Arrhenius plot of ITP hydrolysis in aqueous (●), 20% methanol (▲), and 20% glycerol (■) buffers.

When the temperature was below 17°C methanolic solutions inhibited ITP hydrolysis and glycerol slightly stimulated the hydrolysis.

From Table I we see that E_a increases relative to that in neat solutions for the hydrolysis of both nucleotides in both methanol and glycerol media, with the exception of ITP in glycerol. The values for ΔH^* reflect these same data.

Figure 5 is a Dixon plot that shows the inhibition of F_1-catalyzed ATPase activity by ADP in the three solvent systems. Note that the ordinate for the 20% glycerol data is compressed ten times relative to the aqueous and 20% methanol plots. The relative K_i's for the ADP in each of the solvent systems are calculated to be 1.3, 1.5, and 0.7 mM for the aqueous, methanol, and glycerol systems respectively. These results imply that ADP is a more powerful inhibitor in the presence of 20% glycerol, but a weaker inhibitor in 20% methanol, at the single substrate level tested.

Table I. Tabulation of Thermodynamic Parameters for F_1-catalyzed ATP and ITP Hydrolysis Calculated from Figs. 3 and 4

Solvent	Substrate	E_a (*kJ mol*$^{-1}$)	ΔH^* (kJ mol^{-1})
H_2O	ATP	11	9
20% MeOH	ATP	39	37
20% Gly	ATP	22	19
H_2O	ITP	19	17
20% MeOH	ITP	64	61
20% Gly	ITP	10	7

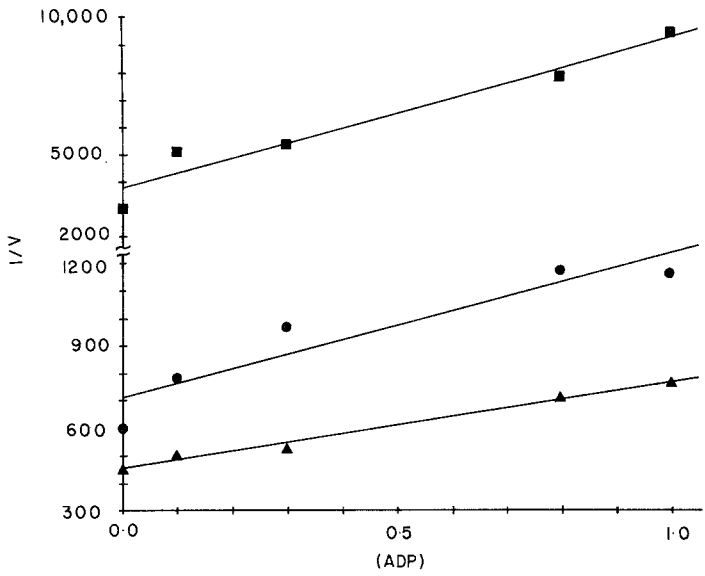

Fig. 5. A Dixon plot of ADP (concentrations are millimolar) acting as an inhibitor in the aqueous (●), 20% methanol (▲), and 20% glycerol (■) systems. Note that the plot of the 20% glycerol data is compressed 10 times relative to the other two plots, thus making the slope appear 10 times shallower than it actually is relative to the aqueous and methanolic systems.

It must be emphasized that the relative inhibition constants reported here were determined using only one substrate concentration. The resulting apparent K_i's are only intended to compare the relative potency of ADP as an inhibitor under the conditions tested.

Discussion

The Arrhenius energies of activation and H^* represent the largest energy transition in the forward direction of the multistep reaction

$$E + ATP \rightleftharpoons E\,ATP \rightleftharpoons E \cdot ATP^* \rightleftharpoons E \cdot ADP \cdot P_i$$
$$\rightleftharpoons E \cdot ADP \cdot P_i^* \rightleftharpoons E + ADP + P_i$$

The reaction sequence is even more complicated if hydrogen ion effects, divalent cation binding, and order of product release are considered. The rates observed are based upon the rate of release of ADP from the enzyme.

The energies of activation that we have calculated are in the range of 10 to 40 $kJ\ mol^{-1}$. Even larger activation energies have been reported for the membrane-bound F_1 from rat liver and Morris Hepatoma 3924A by Melnick

et al. [24]. Lenaz et al. [25] have found that the activation energy of the membrane-bound F_1 increases threefold in the presence of *n*-butyl alcohol.

The kinetic effects observed in the presence of glycerol may be due to several factors. Evidence has been presented previously for a regulatory site on the F_1 complex. In the presence of glycerol these effects disappear. This change in kinetic behavior is completely reversible. Therefore we may postulate that the regulatory site is inhibited in exerting its regulatory power in the presence of glycerol. Another polyhydroxy compound, glucose, also inhibits F_1-ATPase activity [26]. Another possibility is that the effects observed result from the altered viscosity of the co-solvent systems. Somogyi et al. [27] presents a discussion on the influence of viscosity upon apparent thermodynamics and kinetics of enzymatic reactions. That paper predicts that dissociation of the enzyme–product complex is slowed in increasingly viscous solutions. This appears not to be the case for our studies, however. The viscosities of 20% methanol and 20% glycerol are nearly identical at 30°C and are approximately twice that of purely aqueous solution [28]. It is apparent from Figs. 4 and 5, however, that the rate of hydrolysis at 30°C is different for the two organic co-solvents used. This would appear to rule out the hypothesis that our observations are caused by viscosity changes.

Glycerol is used as a co-solvent by other workers in order to stabilize the enzyme against the cold lability inherent in F_1, when dispersed in a purely aqueous buffer [4]. It is now apparent that the presence of glycerol greatly affects the kinetics observed when studying the enzyme. It could cause this alteration by increasing the observed energy of activation, as demonstrated by our data.

Figure 5 demonstrates that product inhibition of the ATPase reaction is also strongly dependent on the solvent system used. These data suggest that the rate of product release can be affected by the solvent used and that product release is the rate-limiting factor in the reaction. This lends further support to other evidence of a similar nature previously proposed by Boyer and co-workers [14–17].

Buffer containing 20% methanol and 0.2 M sucrose has been shown to stimulate greatly the ATPase activity of F_1 while inhibiting the hydrolysis of ITP [10]. When used as a co-solvent in the study of a number of enzymes other than F_1, it was found to have a minimal activating effect [18, 19]. It could be hypothesized that in an hydrolysis reaction, such as that catalyzed by F_1, addition of methanol produced an apparent increase in the activity by participating in alcoholysis. We did not believe this to be the case. First, ethanol, which would be expected to be more nucleophilic than methanol, thus producing a greater alcoholysis effect, in fact produced an optimum stimulation of F_1-ATPase activity approximately half that found with methanol. Second, one would expect increasing methanol concentration to have

produced a proportional increase in apparent activity. This has been demonstrated not to be the case by Schuster [10]. An optimum concentration of 20% (v/v) existed above which activity decreased, returning to control levels at approximately 40% (v/v) methanol. Third, if alcoholysis occurred by the nucleophilic alcohol attacking the electrophilic phosphorus of a phosphate, the hydrolysis of ITP should also have followed a similar increase in activity with increasing methanol concentrations. Such was not the case. A more thorough discussion of the effects of methanol upon F_1-ATPase activity has been presented previously [10].

Although there is little doubt that the solvent used in the study of an enzymatic reaction *in vitro* can greatly modify the observed results, there is no consensus as to which system, if any, closely approximates the actual *in vivo* conditions. Further study of the effects of various organic solvents and their similarity to actual conditions in the mitochondria may reveal new aspects of the control and mechanism of the mitochondrial ATPase.

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