Stimulation of Rat Liver Mitochondrial Adenosine Triphosphatase by Anions

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SUMMARY

The hydrolysis of MgATP by isolated rat liver mitochondrial ATPase (EC 3.6.1.3) at pH 8.0 was stimulated by various anions. The rate of hydrolysis was increased from 18 to 170 μmol per min per mg, a 9.4-fold stimulation, by HSeO₃⁻ at 1 mM MgATP. In the absence of a stimulatory anion, reciprocal plots of initial velocity studies with MgATP as the variable substrate were curved (Hill coefficient approximately 0.5). With the addition of anion, the reciprocal plots became linear. When the substrate was MgITP or MgGTP with the isolated enzyme or MgATP with submitochondrial particles, no curvature of the reciprocal plots was observed. With purified ATPase, anions stimulated the hydrolysis of MgITP, MgGTP, MgUTP, or MgCTP only slightly. With submitochondrial particles the stimulation by anions of MgATP hydrolysis was limited to approximately 2-fold. These data are interpreted to indicate the existence of two substrate sites for MgATP and an anion-binding site on the isolated enzyme.

During the past decade, it has become increasingly apparent that anions affect the properties of numerous enzymes. Particularly prominent among these are ATPases. Both stimulatory and inhibitory effects of various anions on ATPase activity have been reported for rat liver mitochondria (1), rat liver (2, 3) and beef heart (4) submitochondrial particles, isolated ATPase from beef heart (5), rat liver (6,7) and yeast (8) mitochondria, coupling factor 1 from chloroplasts (9), and various microsomal ATPases from pancreas (10), gastric mucosa (11-13), and salivary gland (14). Although the degree of stimulation or inhibition and the specificity differ among these preparations, there appear to be a considerable number of similarities which suggest a common mechanism of action.

In this study the influence of various anions on the ATPase activity of isolated mitochondrial ATPase and submitochondrial particles from rat liver was investigated. The effects of these anions on the hydrolysis of MgATP, MgGTP, MgITP, MgCTP, and MgUTP using the isolated enzyme are compared. Also the influence of anions on the hydrolysis of MgATP by submitochondrial particles was compared to that using the isolated enzyme. The results suggest the existence of two substrate sites, at least for MgATP, and of an anion-binding site.
reaction was followed by observing the disappearance of NADH at 340 nm (315 nm with chromate, 300 nm with 2,4-dinitrophenolate) with a Beckman DU modified with a Gilford model 2220 adapter and a Hewlett-Packard 7101B strip chart recorder.

Linear double reciprocal and Hill plots were constructed from the data using a weighted least squares fit. The weighting factor was the reciprocal of the variance (17). The fold activation and $K_A$ for an anion were determined as follows: using 1 mM MgATP, plots of $1/(v - v_0)$ versus $1/concentration$ of active anion species (where $v =$ velocity in the presence and $v_0 =$ velocity in the absence of anion) were constructed (Fig. 1); the $y$ intercept represented $1/(V_{max} - v_0)$ and fold activation was defined as $V_{max}/v_0$; slope/$y$ intercept = $K_A$. For an anion were determined as follows: using 1 mM MaATP.

First, at pH 8.0, there were several anions that were completely, in one ionic form. These included $SO_3^{2-}$, maleate, $2,4$-dinitrophenolate, and acetylenedicarboxylate. Second, the basis for selection of HSO$_3^-$, HS$O_5^-$, and HP$O_3^-$ was that the activation by sulfite at various pH values (data not shown) corresponded more closely to the concentration of HSO$_3^-$ than to total sulfite or SO$_3^{2-}$ concentration. In the case of malonate and maleate, although at pH 8.0, these anions are almost completely in the dianion form; by varying the pH of the assay system it was shown that the active species was the monoprotonated anion (data not shown). The active species of Na$_2$TeO$_3$ was probably the monoprotonated anion, as in the case of sulfate, but a reliable $pK$ was not determined because of the formation of a precipitate upon titration of Na$_2$TeO$_3$ with acid. The active species of chromate was not determined due to the multiple possibilities including HCO$_3^-$, CrO$_4^{2-}$, and Cr$_2$O$_7^{2-}$. Metasilicate (SiO$_3$) was also found to stimulate MgATP hydrolysis by approximately 2-fold but the active species was not determined because of the difficulty of establishing $pK$ values by titration.

In addition to those anions listed in Table I, numerous others were screened including acetate, arsenate, di-aspartate, N-acetyl-L-glutamate, N-carbamyl-DL-aspartate, chloride, citrate, fumarate, 2,4-glutamate, glutarate, glycolate, iodide, isocitrate, itaconate, di-malate, nitrate, phosphate, phthalate, propionate, salicylate, selenate, sulfate, tartrate, taurine, and tellurate. Several of these anions, e.g., phosphate, arsenate, and sulfate, caused a slight (about 20%) activation at a concentration of approximately 20 mM. In the absence of an activating anion, reciprocal plots of initial velocities with varying MgATP concentration were curved (Hill coefficient approximately 0.5) (Fig. 2). With increasing concentration of an activating anion, the initial velocity of the reaction increased and the reciprocal plots became linear (Hill coefficient 1.0) (Fig. 2). This phenomenon was observed with each of the anions listed in Table I. The differences between anions were: (a) the maximal extent of activation; (b) the anion concentration at which the reciprocal plots became linear; (c) the $K_A$ for the anion; and (d) the $K_m$ for MgATP, which was, for instance, 56, 89, 93, and 117 M in the presence of 10 M HPO$_3^{2-}$, 10 M HCO$_3^-$, 0.14 M HSO$_3^-$, and 0.16 mM maleate, respectively.

**Inhibitory Anions**—Several of the anions examined inhibited the hydrolysis of MgATP. For instance, nitrate caused 50% inhibition of the hydrolysis of 1 mM MgATP at 112 mM in the absence of HCO$_3^-$ and at 20 mM in the presence of 20 mM HCO$_3^-$.

Phosphate, on the other hand, caused slight stimulation of hydrolysis at relatively high MgATP concentrations and slight inhibition at low MgATP concentrations (approximately 30%) inhibition of the hydrolysis of 0.1 mM MgATP at 100 mM phos-
FIG. 2. Initial velocity pattern for activation of MgATP hydrolysis by the indicated millimolar concentrations of HCO$_3^-$.
The numbers in parentheses are the Hill coefficients; standard deviations varied from 0.04 at 0 bicarbonate concentration to 0.1 at 10 mM.

FIG. 3. Inhibition of MgATP hydrolysis by supraoptimal HSO$_4^-$ concentrations; millimolar concentrations are indicated.

phate). In the presence of 20 mM HCO$_3^-$, phosphate produced noncompetitive inhibition versus MgATP ($K_i = 101$ mM, $K_i^* = 11.3$ mM). Considering the activating anions, there appears to be an optimal anion concentration for stimulation. Concentrations above this optimum cause lesser degrees of stimulation and reciprocal plots ($1/v$ versus $1/[\text{MgATP}]$) at these supraoptimal anion concentrations result in patterns analogous to those of noncompetitive substrate inhibition in that both slope and intercept show the inhibitory effect (Fig. 3). It should be noted that the reciprocal plots remain linear even at supraoptimal anion concentrations.

Rat liver mitochondrial ATPase was found to be very sensitive to inhibition by three other anions: $\text{NO}_3^-$, OCN$^-$, and SCN$^-$. In contrast to nitrate and phosphate considered above, these anions produce a greater percentage of inhibition in the absence of activating anions than in the presence of these anions. In the absence of an activating anion, reciprocal plots varying MgATP at different fixed levels of one of these inhibitors resulted in a series of curves with increasing $y$ intercept values (Fig. 4a). The Hill coefficients of these curves were constant with values between 0.5 and 0.6. This was in marked contrast to the situation for activating anions which cause the reciprocal plots to become more linear with increasing anion concentration. In the presence of 10 mM HCO$_3^-$, the pattern of inhibition of these anions was noncompetitive versus MgATP (Fig. 4b). Using 1 mM MgATP and varying HCO$_3^-$ concentration, at different fixed levels of inhibitor, resulted in a competitive pattern of inhibition of these anions versus HCO$_3^-$ (Fig. 5). The various $K_i$ values for these types of experiments with $\text{NO}_3^-$, OCN$^-$, and SCN$^-$ are listed in Table II.
TABLE I

| Inhibitor | $-\text{HCO}_3^-$ | +10 mM HCO$_3^-$ | 1 mM MgATP (K$_{ii}$) |
|-----------|------------------|-----------------|----------------------|
| NaN$_3$   | 2.7 $\mu$m       | 12 $\mu$m       | 4.1 $\mu$m           |
| KOCN      | 29 $\mu$m        | 80 $\mu$m       | 36 $\mu$m            |
| KSCN      | 0.53 mM          | 0.74 mM         | 0.29 mM              |

* $K_{ii}$ values in the absence of HCO$_3^-$ could not be determined due to the curvature of the double reciprocal plots.
* Inhibition constants with varying HCO$_3^-$.

Table III

| Substrate | $-\text{HCO}_3^-$ | 20 mM HCO$_3^-$ | Stimulation |
|-----------|------------------|-----------------|-------------|
| MgATP     | 27 mmol/min/mg   | 85 mmol/min/mg  | 3.1         |
| MgGTP     | 28 mmol/min/mg   | 46 mmol/min/mg  | 1.7         |
| MgITP     | 51 mmol/min/mg   | 81 mmol/min/mg  | 1.6         |

Hydrolysis of Other Nucleotides—The results of experiments using MgGTP, MgITP, MgUTP, and MgCTP as substrates for hydrolysis are in marked contrast to those described above for MgATP. Although anions, e.g. HCO$_3^-$, caused an accelerated rate of hydrolysis of these nucleotides at approximately the same concentrations as with MgATP, the maximum stimulation observed using 20 mM HCO$_3^-$ was 20 to 40% at 1 mM Mg-nucleotide. At $V_{\text{max}}$ for MgGTP and MgITP, 20 mM HCO$_3^-$ produced 60 to 70% stimulation of hydrolysis. Also, in the absence of an activating anion there was no evidence of curvature in the reciprocal plots (Fig. 6). Table III contains a comparison of $K_m$ and $V_{\text{max}}$ values for MgATP, MgGTP, and MgITP with and without 20 mM HCO$_3^-$.

The presence of 1 mM adenosine or AMP did not inhibit the hydrolysis of 1 mM MgITP with or without HCO$_3^-$; the limited extent of HCO$_3^-$ enhancement was not altered.

In addition to being less susceptible to stimulation by anions, MgITP hydrolysis was also less sensitive to inhibition by N$_3^-$

and OCN$^-$ compared to MgATP. The patterns of inhibition of these anions versus MgITP were also different from those versus MgATP. Without HCO$_3^-$ present, N$_3^-$ and OCN$^-$ are non-competitive inhibitors of MgITP hydrolysis (Fig. 7a), while with 20 mM HCO$_3^-$, they appeared to be competitive versus MgITP (Fig. 7b). The various $K_i$ values for N$_3^-$ and OCN$^-$ versus MgITP are listed in Table V.

Anion Effects on ATPase of Submitochondrial Particles—The hydrolysis of MgATP by submitochondrial particles differed in certain respects from that of the isolated ATPase. HCO$_3^-$ and HSO$_4^-$ stimulated the hydrolysis of MgATP by submitochondrial particles at concentrations comparable to those affecting isolated ATPase, but the extent of this stimulation was only about 2-fold. Without anion present, reciprocal plots varying MgATP showed no curvature (Fig. 8). The $K_m$ for MgATP was 50 $\mu$m without anion and 67 $\mu$m with 20 mM HCO$_3^-$.

The inhibition by phosphate of MgATP hydrolysis by submitochondrial particles was competitive versus MgATP in the absence of HCO$_3^-$ ($K_i = 49$ mM) (Fig. 8a) and noncompetitive in the presence of 20 mM HCO$_3^-$ ($K_{ii} = 96$ mM, $K_{i} = 21$ mM) (Fig. 8b).

DISCUSSION

The results of experiments with isolated ATPase using MgATP as the substrate, specifically those in the absence of an activating
anion which result in curvature of the reciprocal plots, suggest the existence of two substrate sites for MgATP. There are several alternative explanations for this type of interaction that would account for these observations, none of which would be easily distinguishable from purely kinetic experiments. If both substrate sites were active in the sense of having hydrolytic activity, there are two possibilities. One would be the existence of two independent active sites which have different kinetic parameters (23). Interestingly, such a proposal has been made in the case of myofibrillar ATPase (24). The other would invoke the existence of two equivalent sites which are interdependent (negative cooperativity) (23, 25). Another alternative would be the existence of only one active site and a separate modifying site (26, 27). Several studies have been reported concerning the number of nucleotide binding sites of the isolated ATPase. Hillborn and Hammes (28) suggest the existence of one “tight” and one “loose” binding site for ADP on beef heart F1, but report less than one ATP bound per mol of F1, probably due to the absence of Mg2+ in their experiments. Harris et al. (29), also using beef heart F1, observed five tight binding sites, three specific for ATP and two for ADP. Garrett and Peneffsky (30) and Peneffsky (31) using beef heart F1 have recently reported the presence of two binding sites for adenylyl imidophosphate, an analog of ATP, as well as the possibility of an additional binding site for ADP. With rat liver mitochondrial ATPase, Catterall and Pedersen (32) find only one ADP binding site. Senior (33) using beef heart F1, have recently reported the presence of two binding sites for adenylyl imidophosphate, an analog of ATP, as well as the possibility of an additional binding site for ADP. With rat liver mitochondrial ATPase, Catterall and Pedersen (32) find only one ADP binding site. Senior (33) suggests that this site may be equivalent to the “tight” binding site of Hillborn and Hammes and that the experimental conditions of Catterall and Pedersen were not adequate to detect a second site with a much larger dissociation constant. Such studies may or may not be relevant to the findings in this paper but they do suggest the possibility of multiple nucleotide binding sites.

The absence of curvature in the reciprocal plots of MgGTP and MgITP suggests that if two sites are present on the isolated enzyme, one of the sites is specific for MgATP or that with MgGTP and MgITP, they are both equivalent and have no interdependence. Since reciprocal plots using MgATP with sub-mitochondrial particles are also linear, the existence of only one nucleotide site or two equivalent, noninteracting sites would also have to be favored in this system.

The effects of the various anions on these systems is also intriguing. The marked activation by these anions of MgATP hydrolysis using isolated ATPase suggests the existence of an anion-binding site. Considering the anions that cause activation (Table I) and those that produce little or no activation, one could suggest a number of size and structural characteristics that would be required for activation and would also determine the extent of activation. The anions that stimulate ATPase are of three basic structures: (a) pyramidal, HSO3-, HSeO3-, HPO32-, HCO3-, HC03, and D(0H)2; (b) tetrahedral, chromate, and SO42-; and (c) dicarboxylic acids, maleate, malonate, and hydroxybenzene. The only activating species which do not fit into these groups are Br- and 2,4-dinitrophenol, although, in the latter case, the structure around the 2-nitro and the ionized —OH is analogous to the structure of the active species of malonate. Size also appears to be one of the criteria for activation as can be seen by comparing the extent of activation and the Ks values of those anions that are included in the pyramidal group above. Interestingly, nitrate inhibits rather than activates mitochondrial ATPase. This singly charged anion is of comparable size but lacks the second, protonated acid group found in most of the anions in this group that activate ATPase. Activation by Br- and not by I- or Cl- supports the importance of size. On the basis of Ks, there appears to be some enhancement of binding afforded by the additional carboxyl group on maleate and malonate as compared to HCO3-. This suggests that there are two binding sites, one that accepts a negatively charged group and the second which accepts a protonated, in this case, carboxyl group since the data indicate the active species of malonate and maleate is the monoprotonated anion. The lack of stimulation by monocarboxylic acids (formate, acetate, propionate) supports the existence of a second binding site for anions. A further degree of structural specificity is suggested by the ineffectiveness of various dicarboxylic acids to stimulate hydrolysis (fumarate, maleate, glutarate). At pH 7.0, 20 mM succinate increased the rate of hydrolysis of 1 mM MgATP by 40%. At pH 8 and 9 succinate did not stimulate significantly. This is consistent with the active species of dicarboxylic acids being the monoprotonated anion for the pKs of maleic acid is 5.64 (21).

The situation with sub-mitochondrial particles would appear to be somewhat different. Although the structural requirements for activation are, for the most part, retained, other factors apparently control the extent of activation since HCO3- and HSO3-, which produce a 5.8- and 8.3-fold activation of the isolated enzyme, respectively, produce approximately a 2-fold stimulation.
with sub-mitochondrial particles. The extent of activation with MgGTP and MgITP using the isolated enzyme is also limited. In this situation, although the effective concentrations are comparable to those that activate MgATP hydrolysis, HCO$_3^-$ and HS$_2$O$_3^-$ cause about a 20% stimulation at 1 mM Mg-nucleotide. This limited activation of hydrolysis is also seen with MgCTP and MgUTP.

The action of N$_3^-$, OCN$^-$, and SCN$^-$ can be explained on the basis of their interaction at the postulated anion-binding site. Such interaction would be competitive versus MgATP, Mg$^{2+}$, and arsenate is stimulated by maleate (34). Further experimentation concerning the conformation of the isolated enzyme and any alteration of this in the presence of anion. It is of interest that Nelson and Racker have reported that in 70% dimethylsulfoxide the rate of ATP hydrolysis in the presence of Mg$^{2+}$ and arsenate is stimulated by maleate (34). Further research will also be required to confirm the existence of two sub-strate sites for MgATP and to determine whether they are both active sites or one is a modifying site without hydrolytic activity.

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