Kinetic Studies of Beef Heart Mitochondrial Adenosine Triphosphatase: Interaction of the Inhibitor Protein and Adenosine Triphosphate Analogues†

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ABSTRACT: The method by which the specific mitochondrial ATPase inhibitor protein (F_1I) operates to inhibit the mitochondrial ATPase (F_1) remains an unanswered question. Its distinctive characteristic is that of inhibition of F_1-catalyzed ATP hydrolysis but not of ATP synthesis (Pullman, M. C., & Monroy, G. C. (1963) *J. Biol. Chem. 238*, 3762–3765). This communication describes studies of the interaction of isolated F_1I with F_1 using ATP and ITP as substrates for the F_1-catalyzed hydrolysis reaction. Two types of dual inhibitor studies were pursued, one in which fixed concentrations of F_1I were incubated in the reaction buffer with varying concentrations of either AMP-P(NH)P, CrADP, or CrATP, all of which are competitive inhibitors of ATP hydrolysis. Dixon plots of the results of initial velocity experiments using these dual innibitor combinations show intersecting lines that exhibited positive cooperativity in every case. The second type of dual inhibitor study examined was that involving various combinations of the different nucleotide inhibitors. Cases specifically investigated were CrATP vs. AMP-P(NH)P, CrADP vs. AMP-P(NH)P, and AMP-P(NH)P vs. ADP. In all these cases, Dixon plots show sets of parallel lines indicative of mutually exclusive inhibitors. Results of previous experiments show the existence of two types of nucleotide binding sites on mitochondrial ATPases, one being a regulatory site specific for various adenosine polyphosphates and the other serving as a catalytic site exhibiting broad specificity (Schuster, S. M., Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250*, 7848–7857). The results obtained from the dual inhibitor studies presented here lead to the postulation that there are two binding areas on the regulatory site, one specific for nucleotides and one specific for F_1I. The Dixon plots that were intersecting and showing positive cooperativity are consistent with such a postulation in that both types of inhibitors are allowed to bind at the same time. The data indicate that nucleotides will compete with each other for binding to a regulatory site, since they show mutually exclusive inhibition.

Beef heart mitochondrial ATPase inhibitor protein (F_1I)[1] was first isolated by Pullman & Monroy (1963). It has since been isolated from rat liver mitochondria (Chan & Barbour, 1976), yeast (Satre et al., 1975), and the bacterium *Escherichia coli* (Smith & Sternweis, 1977). Brooks & Senior (1971) have examined F_1I by polyacrylamide gel electrophoresis in $NaDodSO_4$ and determined its molecular weight to be 10 500. Its amino acid composition is known (Brooks & Senior, 1971), and its stability has been characterized. Van de Stadt et al. (1973) have shown F_1I to be a time-dependent, noncompetitive ATPase inhibitor.

The inhibitor protein's distinct characteristic is inhibition of F_1-catalyzed ATP hydrolysis but not F_1-catalyzed ATP synthesis. However, very limited kinetic studies concerning the interaction of F_1I with F_1 have appeared. Therefore, it is essential to characterize, kinetically, the mechanism of action of F_1I on F_1 in order to understand F_1I's specific mode of action.

ATP hydrolysis has been extensively studied in order to understand its regulation. It has become apparent that the presence or absence of various anions can affect the properties of beef heart ATPase (Pedersen, 1976a; Ebel & Lardy, 1975; Mitchell & Moyle, 1971). The degree of stimulatory or inhibitory effects of these anions on ATPase as well as ITPase activity have been reported (Ebel & Lardy, 1975; Schuster et al., 1975a). The requirement of a sulfhydryl group on F_1 for an anion effect has also been reported (Pedersen, 1976b).

Another avenue studied has been ATPase regulation by the use of nucleotide analogues. Work in this area allows kinetic binding assignments to be made tentatively to the two types of sites on F_1 (Schuster et al., 1975a, 1976). The catalytic site seems to exhibit broad specificity for nucleoside triphosphates, while the regulatory site is more specific for adenosine polyphosphates. This communication presents data regarding the kinetic interactions of F_1I with F_1.

Materials and Methods

F_1 was purified from beef heart mitochondria according to the procedures of Knowles & Penefsky (1972). The chloroform extraction method of Beechey et al. (1975) in conjunction with the column purification method of Spitsberg & Blair (1977) was used also.

The F_1 was stored at 5 °C in 3 M ammonium sulfate, 4 mM ATP, and 4 mM EDTA. Before use, an aliquot of the F_1–ammonium sulfate suspension was centrifuged at 10 000 rpm in an SS-34 rotor (Sorvall) for 15 min to remove the $(NH_4)_2SO_4$ (Knowles & Penefsky, 1972; Horstman & Racker, 1970). The precipitate was then resuspended in 0.1 M sucrose at 30 °C. Since only a small fraction of this diluted F_1 was used in each assay, the concentration of the ammonium sulfate was always less than 0.05 mM. Ebel & Lardy (1975) have previously shown that the effect of ammonium sulfate on F_1 becomes significant only with $(NH_4)_2SO_4$ concentrations near 20 mM.

F_1I was isolated according to the procedure of Horstman & Racker (1970) with the exception that the fractionation step using ethanol was deleted. Although this preparation is not

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[1] Abbreviations used: CrATP, chromium(III) adenosine 5′-triphosphate; CrADP, chromium(III) adenosine 5′-diphosphate; AMP-P(NH)P, adenylyl imidodiphosphate; TEA, triethanolamine; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; F_1, beef heart mitochondrial ATPase; F_1I, beef heart mitochondrial ATPase inhibitor protein.

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completely pure, no interfering activities were found. Protein concentrations were determined by a Biuret method (Layne, 1957) with crystalline bovine serum albumin used as a standard.

The chromium(III) complexes of ADP and ATP were prepared as described by Danenberg & Cleland (1975) and DePamphilis & Cleland (1973). The concentrations of CrADP and CrATP were determined from extinction coefficients as described in the above papers.

ATPase activity was assayed at 27 °C in a total volume of 1 mL containing 50 mM TEA-Cl, pH 8.0, 200 mM sucrose, 6 mM $MgCl_2$, 2 mM KCl, 1.34 mM phosphoenolpyruvate, 0.3 mM NADH, and 8 μg each of pyruvate kinase and lactate dehydrogenase. All reactions were started with the addition of 0.02 mg of F_1 and then monitored by observing the disappearance of NADH absorbance at 340 nm with a Beckman DU monochromator, Gilford Model 2220 adapter, and Hewlett-Packard 7101 B strip chart recorder. Enzyme reactions were monitored until a steady-state rate was obtained. Due to the slow interaction of F_1 and F_1I, this often took between 10 and 20 min. The nucleotides and analogues used in these studies have been shown previously not to interfere with the coupled enzyme assay (Schuster et al., 1975b).

The studies to determine the inhibitory effect of F_1I on F_1 with ATP and ITP as substrates were performed at 27 °C in a total volume of 1.0 mL. The assay buffer was the same as described above. ATP concentrations varied from 0.1 mM to 2 mM, and ITP concentrations ranged from 0.2 mM to 5mM. The nucleotide, F_1I, and buffer were incubated together for 15 min and the reaction was then started by the addition of ATPase and monitored as described above. In all cases linear rates were used in the data presented. This allowed for steady-state approximations to be made after the inhibitors reached equilibrium with the enzyme (Penefsky, 1974; Schuster et al., 1975a).

Dual inhibitor studies using ATP as the substrate and monitoring inhibition of F_1I and AMP-P(NH)P on ATPase activity were performed by using the above conditions. Dual inhibitor studies involving the chromium(III) complexes of ADP and ATP were performed at 27 °C in a total volume of 1.0 mL; 50 mM BES, pH 7.6, was used instead of TEA-Cl. The inhibition of F_1 monitored by using ADP and AMP-P-(NH)P was done by using a phosphate assay (Schuster et al., 1976). The assay buffer contained 3 mM ATP, 6 mM Mg^{2+}, 60 mM TEA-Cl, pH 8.0, and 0.25 M sucrose.

All compounds were purchased from common commercial suppliers and were of the highest purity available.

Results

Previous studies of the F_1I inhibitor protein have focused upon the isolation (Pullman & Monroy, 1963; Chan & Barbour, 1976; Satre et al., 1975; Smith & Sternweis, 1977) and chemical characterization (Brooks & Senior, 1971) while only limited kinetic studies concerning the interaction of F_1 with F_1I have appeared (Van de Stadt et al., 1973). Van de Stadt et al. (1973) have shown F_1I to be a time-dependent, noncompetitive ATPase inhibitor assuming that the hydrolysis of ATP by F_1 obeys simple Michaelis–Menten kinetics. However, the studies presented in this paper indicate that the interaction of F_1 with F_1I is more complex than simple Michelis–Menten kinetics can represent.

Ebel & Lardy (1975) and others (Pedersen, 1976a) have shown that the ATP hydrolysis catalyzed by beef heart F_1 shows marked negative cooperativity. It was further shown that the kinetics of the hydrolysis of ATP is controlled by the presence of various anions (Pedersen, 1976a; Ebel & Lardy, 1975; Mitchell & Moyle, 1971) and that the hydrolysis of inosine and guanosine triphosphate exhibit markedly different kinetics. The effect of F_1I on beef heart mitochondrial ATPase activity is shown in Figure 1. The negative cooperativity that is present without F_1I in the absence of activating anion remains unchanged at all levels of F_1I tested. In addition, it appears that even at high ATP concentration, the F_1I inhibition of ATP hydrolysis persists.

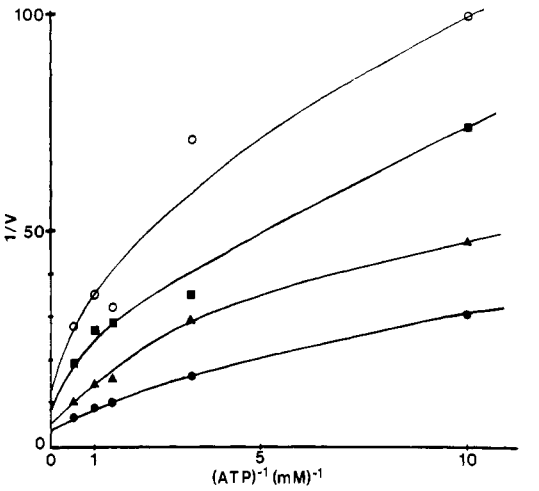


FIGURE 1: Effect of F_1I on beef heart mitochondrial ATPase activity. Velocities are plotted as the reciprocal of nanomoles of product per minute per milligram of protein. The micromolar concentrations of F_1I are as follows: (●) 0, (▲) 0.107, (■) 0.214, (○) 0.428.

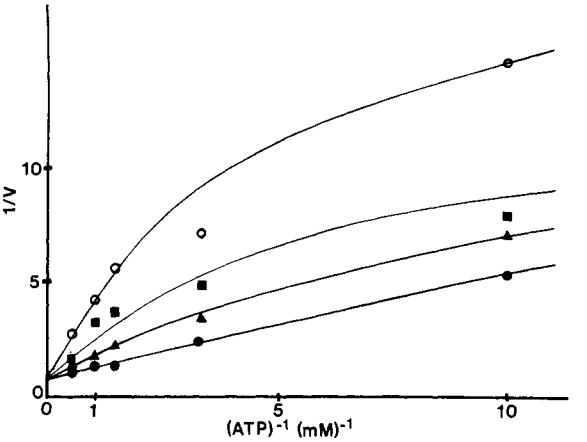


FIGURE 2: Effect of F_1I on beef heart mitochondrial ATPase activity in the presence of 20 mM $NaHCO_3$. Velocities are plotted as the reciprocal of nanomoles of product produced per minute per milligram of protein. The micromolar concentrations of F_1I are as follows: (●) 0, (▲) 0.107, (■) 0.214, (○) 0.428.

Both stimulatory and inhibitory effects of anions on F_1 activity have been reported (Ebel & Lardy, 1975; Schuster et al., 1975a) when ATP is the F_1 substrate. Ebel & Lardy (1975) and Pedersen (1976a) have shown that an increased concentration of certain activating anions such as bicarbonate increased the initial velocity of the ATP hydrolysis reaction and this resulted in a removal of the negative cooperativity seen without such anions. In light of this, it was deemed necessary to monitor the effects of F_1I on the F_1-ATPase activity in the presence of such activating anions. Figure 2 shows the effect of fixed concentrations of F_1I on F_1-catalyzed ATP hydrolysis in the presence of 20 mM $NaHCO_3$. As shown in Figure 2, the presence of 20 mM HCO_3^- has eliminated the negative cooperativity of ATP hydrolysis seen before (Figure 1) in the absence of activating anions or inhibitor. Under conditions of 20 mM HCO_3^- in the assay, the F_1I still inhibits ATPase activity, but the potency has decreased. In

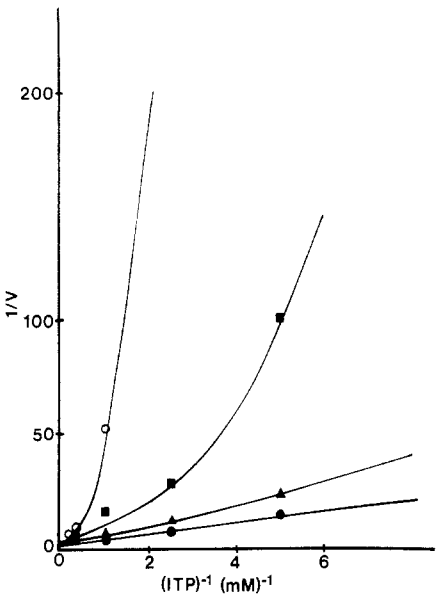


FIGURE 3: Effect of F_1I on beef heart mitochondrial ITPase activity. Velocities are plotted as the reciprocal of nanomoles of product produced per minute per milligram of protein. The micromolar concentrations of F_1I are as follows: (●) 0, (▲) 0.107, (■) 0.214, (○) 0.428.

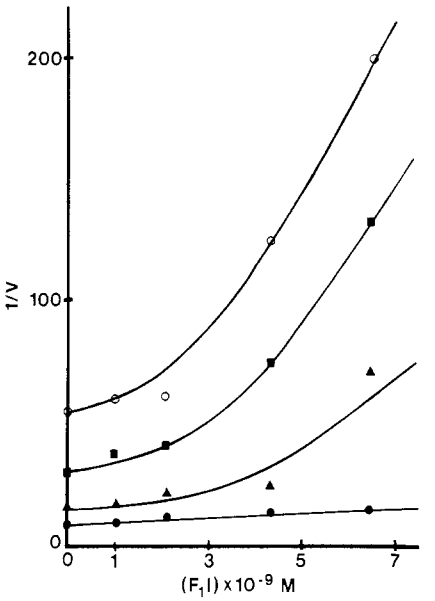


FIGURE 4: Effect of varying concentrations of F_1I at fixed AMP-P-(NH)P concentrations on beef heart mitochondrial ATPase activity. Velocities are plotted as the reciprocal of nanomoles of product produced per minute per milligram of protein. The micromolar concentrations of AMP-P(NH)P are as follows: (●) 0, (▲) 1.0, (■) 2.0, (○) 3.0.

addition, as the F_1I concentration is increased, the negative cooperativity reappears (see Figure 2). The most striking difference between the effect of F_1I on F_1-ATPase activity with or without HCO_3^- is the apparent competitiveness of the F_1I inhibition when HCO_3^- is present.

We have previously proposed a regulatory scheme for the control of F_1 hydrolytic activity (Schuster et al., 1975a). From this proposal, it appears there are two types of sites on the F_1. One of these is a catalytic site that has a broad range of specificity for nucleoside triphosphates, and the other is thought to be a regulatory site specific for only adenosine polyphosphates. Evidence indicates that other nucleoside polyphosphates may bind to the proposed regulatory site, but adenine nucleotide binding to the regulatory site is essential for causing an inhibition of activity at the catalytic site (Schuster et al., 1976). The relation of F_1I to these different sites has not been explored.

As previously described, the F_1-catalyzed hydrolysis of ATP and ITP exhibit markedly different kinetics (Pedersen, 1976a; Ebel & Lardy, 1975; Mitchell & Moyle, 1971; Schuster et al., 1975a; Pedersen, 1976b). For example, the hydrolysis of ITP was in part studied by using an ITP analogue, IMP-P-(NH)P. Schuster et al. (1976) have shown that IMP-P(NH)P is a potent competitive inhibitor of ITP hydrolysis and that marked positive cooperativity was apparent. ATP hydrolysis was only inhibited by IMP-P(NH)P when the substrate concentration was low. When it was high, activation was observed. Therefore, the ATPase inhibitors must be studied with more than one substrate in order to understand fully the nature of their action on F_1 activity. Figure 3 shows the result of using F_1I with ITP as the varied substrate. Two very distinct effects were obtained, the first being a marked positive cooperativity as the F_1I is increased, and the second being an apparent competitiveness of F_1I inhibition with ITP as the substrate.

The results thus far presented indicate that the inhibition of F_1 hydrolytic activity occurs via a complex process. Since previous work has established kinetic assignments for the binding of nucleotides to the catalytic and regulatory sites of F_1, it was deemed essential to establish the relationship of F_1I to these sites. By observing the effects of pairs of F_1 inhibitors on the inhibition patterns for ATP hydrolysis, we felt we could designate whether or not F_1I was binding exclusively or in an interacting mode with other inhibitors (see Discussion).

In order to accumulate this information, two types of dual inhibitor studies were done, and in all cases, F_1-catalyzed ATP hydrolysis was measured. The first type of study involved combinations of F_1I and three different nucleotide analogues. The analogues were AMP-P(NH)P, CrADP, and CrATP. The second type of dual inhibitor study utilized combinations of the ATP analogues, namely CrADP and AMP-P(NH)P, CrATP and ADP.

AMP-P(NH)P is a potent competitive inhibitor of beef heart mitochondrial ATPase (Schuster et al., 1975a; Penefsky, 1974). The result of its action is very similar to that of F_1I in that it does inhibit ATP hydrolysis but has no inhibitory effect on ATP synthesis. Figure 4 shows a Dixon plot of the inhibition of F_1 by F_1I and AMP-P(NH)P. Positive cooperativity becomes evident as the concentration of AMP-P(NH)P increases.

The effect of the chromium(III) complexes of both ADP and ATP on beef heart mitochondrial ATPase has been investigated (Schuster et al., 1975b). CrADP and CrATP were found to be competitive inhibitors of ATP hydrolysis since they compete for binding at the same site as ATP (Schuster et al., 1975b). Figure 5 shows the effect of CrADP at fixed F_1I concentration on F_1-ATPase activity. Although not shown, the effect of CrATP on F_1-ATPase activity in the presence of F_1I is similar to the effects seen in Figure 5 for CrADP. The Dixon plots in both cases show nonlinear, nonparallel plots. As the concentration of F_1I increases, positive cooperativity is more pronounced in both cases.

It appears obvious from the above three experiments that the two inhibitors involved in each case are interacting inhibitors. In this situation, the two inhibitors bind to the enzyme at different sites and the binding of either inhibitor prevents the substrate from being converted to product. The two inhibitors may compete for different portions of the substrate binding site or they may combine with the enzyme at specific sites in such a way as to distort the substrate binding site. The

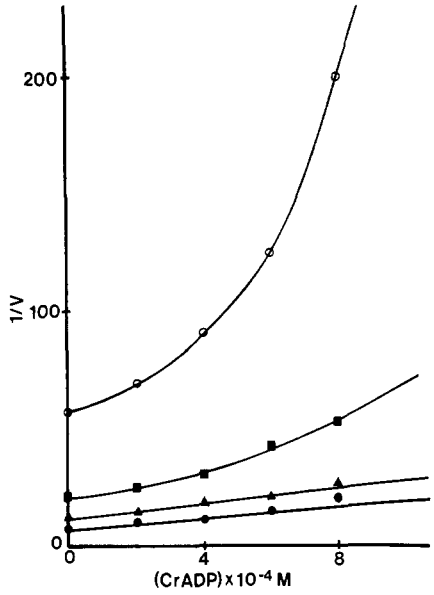


FIGURE 5: Effect of varying concentrations of CrADP at fixed F_1I concentrations on beef heart mitochondrial ATPase activity. Velocities are plotted as the reciprocal of nanomoles of product produced per minute per milligram of protein. The micromolar concentrations of F_1I are as follows: (●) 0, (▲) 0.107, (■) 0.214, (○) 0.428.

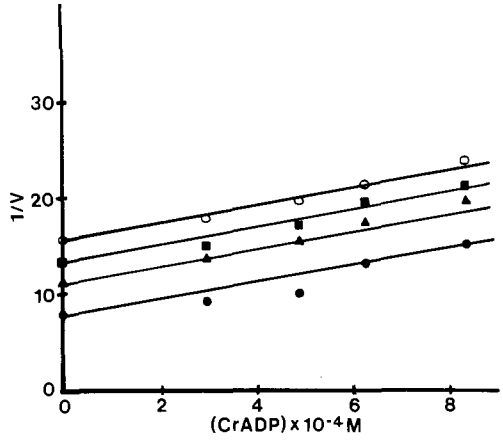


FIGURE 6: Effect of varying concentrations of CrADP at fixed AMP-P(NH)P concentrations on beef heart mitochondrial ATPase activity. Velocities are plotted as the reciprocal of nanomoles of product produced per minute per milligram of protein. The micromolar concentrations of AMP-P(NH)P are as follows: (●) 0, (▲) 20.0, (■) 40.0, (○) 60.0.

positive cooperativity present in all three cases indicates that the binding of F_1I is possibly enhancing the inhibition of ATP hydrolysis of the bound nucleotide.

The experiments done using the inhibitor pairs of the ATP analogues yielded strikingly different results from the previous dual inhibitor studies. In all three cases, CrADP and AMP-P(NH)P (Figure 6), CrATP and AMP-P(NH)P (Figure 7), and AMP-P(NH)P and ADP (Figure 8), Dixon plots show sets of parallel lines. The sets of parallel lines indicate that the enzyme can combine with either inhibitor but not simultaneously with both. Thus the two inhibitors are mutually exclusive with respect to each other.

Discussion

It has been previously shown that ATP hydrolysis catalyzed by beef heart F_1 (Schuster et al., 1975a) and rat liver F_1 (Pedersen, 1976a) showed marked negative cooperativity. In the presence of some activating anions such as HCO_3^-, it appeared that the initial velocity of ATP hydrolysis was increased and that this resulted in a removal of the negative cooperativity. Figures 1 and 2 represent data that are consistent with these two observations. As regards the F_1I, in the absence of 20 mM HCO_3^-, F_1I does appear to be a noncompetitive ATPase inhibitor. However, in the presence of HCO_3^- the F_1I is a competitive inhibitor of ATP hydrolysis.

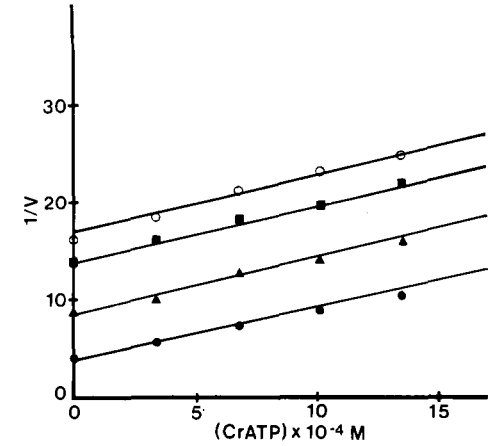


FIGURE 7: Effect of varying concentrations of CrATP at fixed AMP-P(NH)P concentrations on beef heart mitochondrial ATPase activity. Velocities are plotted as the reciprocal of nanomoles of product produced per minute per milligram of protein. The micromolar concentrations of AMP-P(NH)P are as follow: (●) 0, (▲) 40.0, (■) 60.0, (○) 80.0.

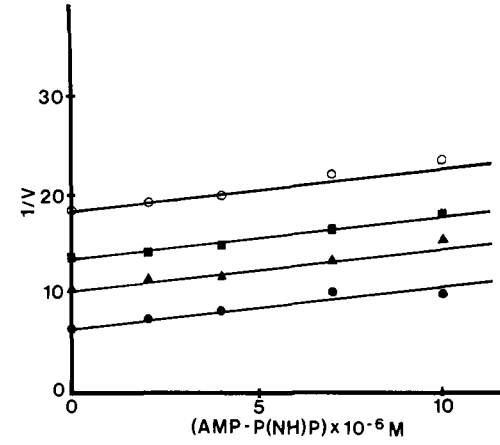
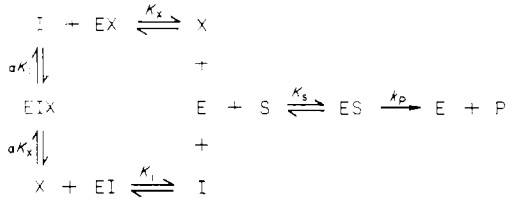


FIGURE 8: Effect of varying concentrations of AMP-P(NH)P at fixed ADP concentrations of beef heart mitochondrial ATPase activity. Velocities are plotted as the reciprocal of nanomoles of product produced per minute per milligram of protein. The micromolar concentrations of ADP are as follows: (●) 0, (▲) 20.0, (■) 40.0, (○) 70.0.

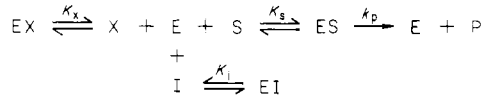
Previous studies indicate that the hydrolyses of ATP and ITP exhibit strikingly different kinetics (Pedersen, 1976a; Ebel & Lardy, 1975; Mitchell & Moyle, 1971; Schuster et al., 1975a). This is also evident in comparing the data presented here (Figures 1 and 3). With ITP as the varied substrate, marked positive cooperativity is present with increasing fixed concentrations of F_1I. The F_1I inhibition of F_1-ITPase activity appears to be competitive. It is obvious that the inhibition of F_1-catalyzed ATP hydrolysis by the inhibitor protein is much more complex than previously thought.

Multiple inhibitor studies can yield a great deal of information about the different binding sites on an enzyme and how they interact. It is not known how or when F_1I binds to F_1 to inhibit ATP hydrolysis. Previous studies (Pullman & Monroy, 1963; Horstman & Racker, 1970) have shown that the F_1I inhibition of ATPase activity is dependent upon the presence of ATP, yet the nature of this dependency is not understood. Many other ATPase inhibitors have been studied

Scheme I



Scheme II



more extensively, and their interaction with beef heart ATPase is proposed (Schuster et al., 1975a). It was hoped that by utilizing some of the many highly documented inhibitors in conjunction with F_1I, a better understanding of the inhibitor binding sites on F_1 would be gained. For example, we could tell whether there were multiple sites for a given inhibitor, a single site capable of binding different inhibitors, or multiple sites each specific for a different inhibitor.

Dual inhibitor studies can be interpreted by postulating binding mechanisms with their relevant kinetic equations to test the results of various experiments. The inhibition patterns of F_1-catalyzed ATP hydrolysis seen in the presence of various inhibitor pairs presented in this communication can be most simply interpreted by postulating binding mechanisms for mutually exclusive inhibitor pairs and those for interacting inhibitors. Various schemes are useful in explaining the markedly different results obtained between the dual inhibitor studies using nucleotide analogue pairs and those inhibition patterns representative of the various combinations of F_1I and a nucleotide analogue.

The simplest scheme that fits the inhibition patterns of the interacting inhibitors was that described by Segel (1975) for cooperative pure competitive inhibition by two different nonexclusive inhibitors. The binding mechanism is given in Scheme I, where k_s, K_x, K_i, αK_x, and αK_i are the dissociation constants for ES, EX, EI, and EIX, respectively. The velocity equation for this scheme in Dixon plot form is given by eq 1.

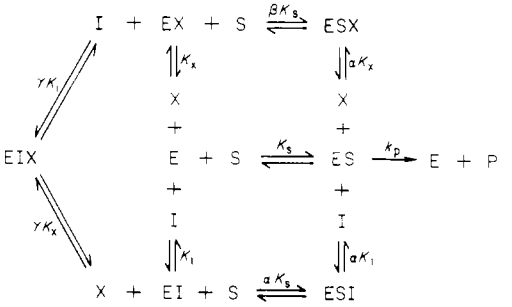
$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_x}\left(1 + \frac{[X]}{\alpha K_x}\right)[I] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]} + \frac{K_s[X]}{K_x[S]}\right) \quad (1)$$

When both inhibitors I and X are present, the degree of inhibition is always greater than that observed for the same total specific concentration of either inhibitor alone. The nonexclusivity of I and X binding can be shown by Dixon plots of $1/V$ vs. the concentrations of one inhibitor at fixed [S] and varied concentrations of the second inhibitor. In both cases ([X] vs. $1/V$ or [I] vs. $1/V$), both the slope and intercept are dependent upon the concentration of the second inhibitor.

If the binding of the competitive inhibitors X and I is mutually exclusive, dramatically different results are obtained. This binding mechanism is represented in Scheme II, where K_s, K_x and K_i are the dissociation constants for ES, EX, and EI, respectively. The velocity equation is given eq 2. Mixtures

$$\frac{V}{V_{max}} = \frac{[S]}{K_s\left(1 + \frac{[X]}{K_x} + \frac{[I]}{K_i}\right) + [S]} \quad (2)$$

Scheme III



of the two inhibitors are competitive with respect to each other. Dixon plots can be used to show either I or X as the varied inhibitor. When [I] is varied, the equation becomes

$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_i}[I] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]} + \frac{K_s[X]}{K_x[S]}\right) \quad (3)$$

When [X] is varied:

$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_x}[X] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]} + \frac{K_s[I]}{K_i[S]}\right) \quad (4)$$

Thus, the plot of $1/V$ vs. [I] at fixed [S] and [X] is linear with a slope that is independent of [X]. Consequently, the family of lines obtained at different fixed concentrations of [X] are parallel. The same is also true of the family of lines obtained when different fixed concentrations of I are plotted. The lines will not be parallel if both I and X combine simultaneously with E.

The competitive ATPase inhibitors ADP, CrADP, CrATP, and AMP-P(NH)P are all mutually exclusive inhibitors. Figures 6–8 clearly show the parallel lines obtained when these inhibitors are used in combination.

The above discussion demonstrates that, for the case of two competitive inhibitors (X and I), when one inhibitor is varied the slope of the Dixon plots can reveal if [X] and [I] bind exclusively or cooperatively. However this requirement of X and I being competitive may not be strictly true in the case of several nucleotides and F_1 or as shown above for F_1I when ATP is the F_1 substrate in the absence of bicarbonate. When the requirement of X and I being competitive is removed, the model becomes that of Scheme III, where K_s, K_x, and K_i are dissociation constants for ES, EX, and EI; αK_s and αK_i are dissociation constants for EIS; βK_s and βK_x are dissociation constants for ESX; and γK_i and γK_x are dissociation constants for EIX, respectively. The velocity equation is given by eq 5. The mutual exclusivity of I and X will be evident from

$$\frac{V}{V_{max}} = \frac{[S]}{K_s\left(1 + \frac{[X]}{K_x} + \frac{[I]}{K_i} + \frac{[X][I]}{\alpha K_x K_i}\right) + [S]\left(1 + \frac{[X]}{\beta K_x} + \frac{[I]}{\alpha K_i}\right)} \quad (5)$$

the Dixon plots of $1/V$ vs. the concentration of one inhibitor at a fixed [S] and various fixed concentrations of the other inhibitor, since

$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_i}\left(1 + \frac{[S]}{\alpha K_s} + \frac{[X]}{\gamma K_s}\right)[I] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]} + \frac{K_s[X]}{K_x[S]} + \frac{[X]}{\beta K_x}\right) \quad (6)$$

or

$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_x}\left(1 + \frac{[S]}{\beta K_s} + \frac{[I]}{\gamma K_i}\right)[X] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]} + \frac{K_s[I]}{K_i[S]} + \frac{[I]}{\alpha K_i}\right) \quad (7)$$

In either case, the slopes are dependent upon the concentration of the second inhibitor if all binding described is significant. However, if γK_x and γK_i are significantly larger then [X] and [I], respectively (i.e., mutual binding of [X] and [I] is highly unfavorable), the family of curves will again be parallel. It is clear that it is not necessary for both [X] and [I] to be competitive inhibitors to interpret parallel lines on Dixon plots as indicating mutually exclusive binding. Indeed, more than one type of inhibition can possibly be taking place, and the same qualitative description will hold.

When F_1I is used as one of the members of the inhibitor pairs, there appears an upwards curvature of the Dixon plots in all the cases examined (Figures 4 and 5). The idea of multiple binding must be included to understand this phenomenon. That is, that after the first molecules of X and I bind to E, there is the opportunity for a second binding of either X or I, pulling the equilibria further from the productive ES complex.

An expansion of the model for two nonexclusive competitive inhibitors described above can also be used to represent interacting inhibitors. If an additional molecule of [I] could bind to the EIX complex with the dissociation constant being K_w and another molecule of X could bind to EIXI with the dissociation constant being K_z, the velocity equation would be

$$\frac{V}{V_{max}} = [S]/\left\{K_s\left(1 + \frac{[I]}{K_i} + \frac{[X]}{K_x} + \frac{[X][I]}{\alpha K_x K_i} + \frac{[X][I][I]}{\alpha K_x K_i K_w} + \frac{[X][I][I][X]}{\alpha K_x K_i K_w K_z}\right) + [S]\right\} \quad (8)$$

After rearrangement of the velocity equation into Dixon plot form, one obtains

$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_i}\left(1 + \frac{[X]}{\alpha K_x} + \frac{[X][I]}{\alpha K_x K_w} + \frac{[X]^2[I]}{\alpha K_x K_w K_z}\right)[I] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]} + \frac{K_s[X]}{K_x[S]}\right) \quad (9)$$

and

$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_x}\left(1 + \frac{[I]}{\alpha K_i} + \frac{[I]^2}{\alpha K_i K_w} + \frac{[I]^2[X]}{\alpha K_i K_w K_z}\right)[X] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]} + \frac{[K_s[I]}{K_i[S]}\right) \quad (10)$$

Again, the nonexclusivity of X and I binding can be shown by the Dixon plots. Both the slope and intercept of $1/V$ vs. [I] and $1/V$ vs. [X] are dependent upon the concentration of the second inhibitor. The slopes in both cases are also dependent upon the varied inhibitor. Thus, these plots will show intersecting curved lines with upwards curvature whether $1/V$ vs. [I] or $1/V$ vs. [X] is plotted.

Again the restriction of the inhibitor being competitive need not limit the discussion. If the above model for two noncompetitive inhibitors X and I is to allow for binding of another molecule of I to the EIX complex with a dissociation constant of K_w and another molecule of X binding to the EIXI complex with a dissociation constant of K_z, the equation for the Dixon plot of $1/V$ vs. [I] becomes

$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_i}\left(1 + \frac{[S]}{\alpha K_s} + \frac{[X]}{\gamma K_x} + \frac{[X][I]}{\gamma K_x K_w} + \frac{[X]^2[I]}{\gamma K_x K_w K_z}\right)[I] + \frac{1}{V_{max}} + \left(1 + \frac{K_s}{[S]} + \frac{[X]}{\beta K_x} + \frac{K_s[X]}{K_x[S]}\right) \quad (11)$$

and $1/V$ vs. [X] becomes

$$\frac{1}{V} = \frac{K_s}{[S]V_{max}K_s}\left(1 + \frac{[S]}{\beta K_s} + \frac{[I]}{\gamma K_i} + \frac{[I]^2}{\gamma K_i K_w} + \frac{[I]^2[X]}{\gamma K_i K_w K_z}\right)[X] + \frac{1}{V_{max}}\left(1 + \frac{K_s}{[S]} + \frac{[I]}{\alpha K_i} + \frac{K_s[I]}{K_i[S]}\right) \quad (12)$$

Again, a plot of either $1/V$ vs. [I] or $1/V$ vs. [X] will produce intersecting, curved lines with upwards curvature. Clearly, these are the simplest models that fit Figures 4 and 5. When F_1I is an inhibitor in the presence of a nucleotide analogue, it appears that additional binding is induced.

Thus, the results of the dual inhibitor studies allowed two important conclusions to be made concerning the interaction of F_1I and nucleotide analogues with beef heart mitochondrial ATPase. First, when F_1I and nucleotide analogues are used as inhibitor pairs of F_1, the two inhibitors are interacting. Figures 4 and 5 show that F_1I with CrADP, CrATP (data not shown), and AMP-P(NH)P give intersecting curved Dixon plots. Both members of the pair are able to bind to the enzyme simultaneously and, in fact, the binding mechanisms and kinetic equations show multiple binding of both types of inhibitors. This probably indicates that F_1I and the nucleotide analogue are binding at two different sites on F_1, F_1I binding to its specific site and the nucleotide analogue binding to the regulatory site specific for adenosine polyphosphates. Secondly, when two nucleotide analogues are used to study the inhibition of F_1, they act as mutually exclusive inhibitors. The Dixon plots (Figures 6–8) and the equilibria presented indicate this fact for the pairs CrADP and AMP-P(NH)P, CrATP and AMP-P(NH)P, and ADP and AMP-P(NH)P. The nucleotide pairs are competing with each other for the same binding site.

We have suggested (Schuster et al., 1975a) that there appears to be two types of binding sites on the F_1. One of these, a catalytic site, has a broad specificity for nucleoside triphosphates, while the other, a regulatory site, seems to be specific for adenosine polyphosphates. The model proposed is an expansion or modification of the previously suggested model that accommodates the data presented in this communication. The modification still suggests the existence of a catalytic site exhibiting broad specificity for nucleotides and a regulatory site that seems more specific for adenosine polyphosphates, but it also proposes a separate binding site in the regulatory area for the binding of F_1I. In addition, the data support the notion that the F_1I binding site lies in very close proximity to the regulatory binding site for the adenosine polyphosphates.

The inhibition of F_1 by F_1I indicates that in the absence of HCO_3^-, both ATP and F_1I bind to their specific sites independently and simultaneously (Figure 1). In the presence of HCO_3^-, though, ATP and F_1I might bind closer together on their respective sites since the double-reciprocal plot (Figure 2) indicates F_1I is a competitive inhibitor of F_1-catalyzed ATP hydrolysis. The presence of HCO_3^- might cause ATP to bind

on its specific site physically closer to the F_1I binding site or the site could be sensitive to F_1I binding. This could account for the fact that F_1I appears competitive in nature; i.e., ATP could displace F_1I at infinite ATP concentrations.

With ITP as the substrate, F_1I appears to be a competitive inhibitor (Figure 3). Again, this seems to suggest that ITP must bind in closer proximity on the regulatory binding site to the F_1I binding site so that ITP could displace F_1I. Previous work (Schuster et al., 1976) suggests that ITP does indeed bind to the F_1 regulatory site, but that such binding does not affect the activity at the F_1 catalytic site. Since the data of Figure 3 indicate that, in the presence of F_1I, ITP hydrolysis proceeds with positive cooperativity, the bound F_1I may cause ITP at the regulatory site to enhance hydrolytic activity at the catalytic site.

The data presented here further suggest separate sites for nucleotide binding and F_1I binding. When F_1I and a nucleotide analogue were used as inhibitor pairs to study inhibition of ATP hydrolysis in all cases (Figures 4 and 5), intersecting and curved Dixon plots were obtained. This allows for the conclusion to be made that the two inhibitors were interacting. Thus, when F_1I and either AMP-P(NH)P, CrADP, or CrATP were paired, the Dixon plots conclusively show that F_1I binds to its specific site and the nucleotides bind to the regulatory site specific for adenosine polyphosphates independently. From these data it also appears that multiple binding occurs, although direct binding studies are necessary to determine the stoichiometry.

When pairs of the nucleotide analogues were used (Figures 6–8), sets of parallel lines were obtained for Dixon plots, indicating that the two nucleotide inhibitors were mutually exclusive. This supports the notion that nucleotides have one regulatory site while F_1I has a distinct binding site. There most likely is an interaction between the F_1I site and the nucleotide binding regulatory site. Since F_1I in combination with all of the nucleotide analogues tested yields nonlinear Dixon plots, the sites might either interact or be very close.

The proposed model is not intended to imply that catalysis and regulation occur in separate subunits, even though such a scheme is possible. Instead, it merely reflects the apparently distinct nature of the catalytic and regulatory sites. Such of model is also useful in trying to explain how F_1I can inhibit F_1-catalyzed ATP hydrolysis but yet not inhibit F_1-catalyzed ATP synthesis (Van de Stadt et al., 1973). If F_1I were binding directly to the catalytic site, such a differential action would seem unlikely. However, by having a separate site, F_1I could allosterically alter the catalytic site so as to make substrate binding or product release more or less favorable. This action could have the overall effect of enhancing the reaction in only one direction.

Acknowledgments

We gratefully acknowledge Lonnie Dorgan for his technical assistance in a portion of this work.

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