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EXPLORING SITES ON MITOCHONDRIAL ATPASE FOR CATALYSIS, REGULATION, AND INHIBITION

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Evidence is presented that mitochondrial ATPase has two types of sites that bind adenine nucleotides. The catalytic site, C, binds the substrates ATP, GTP, or ITP and the inhibitor guanylyl imidodiphosphate (GMP-PNP). A second type of site, R, binds ATP, ADP, adenylyl imidodiphosphate (AMP-PNP), and the chromium complexes of ATP or ADP. All of these substances binding to the R site inhibit the hydrolysis of ATP in a competitive manner; their inhibition of hydrolysis of ITP and GTP is noncompetitive. GMP-PNP inhibits oxidative phosphorylation in submitochondrial particles but AMP-PNP does not. The localization on mitochondrial membranes of sites for the binding of various antibiotics that inhibit oxidative phosphorylation is discussed.

INTRODUCTION

Many different types of experimental approaches have yielded useful information concerning the process of oxidative phosphorylation. Yet despite the impressive array of established facts and inferences drawn therefrom, it is still too early to designate a favored mechanism from among the many available or to exclude any of the more reasonable hypotheses that have been offered. It is a prime time for gathering still more facts; from facts, in due course, a mechanism will be made obvious to all of us.

In our own laboratory we have used a number of different approaches to examine the nature of the ATP synthesis machinery and we will briefly summarize several of these. The first view will be that gained from experiments with antibiotic inhibitors. We will then consider nucleotide analog effects on exchange reactions, the sequence of events in the synthesis of ATP, and finally, the effects of nucleotide analogs on ATPase and on phosphorylating respiration.

The preparation of submitochondrial particles and the assays for ATPase activity have been described elsewhere (1). The ATPase assay involves the continued regeneration of ATP with phosphoenolpyruvate and pyruvate kinase. This ensures that minimal concentrations of ADP, a powerful inhibitor of ATPase, will be present. Pyruvate release is monitored at 340 nm by NADH and lactic dehydrogenase. A 1 mM excess of free Mg^{++} was present in all experiments. Rat liver mitochondrial ATPase was purified by the procedure of Lambeth and Lardy (2); the corresponding heart enzyme was prepared according

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to Knowles and Penefsky (3). Kinetic data are summarized in double reciprocal plots by use of weighted least squares fit; the weighting factor was the reciprocal of the variance (4).

RESULTS

Binding Sites for Antibiotic Inhibitors of Oxidative Phosphorylation

We presented this subject in detail at the Minneapolis meeting of the American Society of Biological Chemists in June of 1974 (5) and the facts can be summarized as shown in Fig. 1.

There are two sites on liver mitochondrial ATPase for binding aurovertin. The presence of ATP does not interfere with the binding of aurovertin but does alter the fluorescence yield of the aurovertin-ATPase complex, indicating an altered conformation of the complex when ATP is bound. The antibiotic A23871 (Efrapeptin) is a powerful inhibitor of the purified mitochondrial ATPase. It appears to compete with ATP. It is a much more effective inhibitor when added to the isolated enzyme before ATP is added. In the presence of ATP, efrapeptin becomes progressively more inhibitory as time goes on.

The oligomycins, venturicidin X and ossamycin share a common inhibitory site that requires a membrane component – oligomycin sensitivity conferring protein. Leucinostatin (A20668) inhibits ATPase in much the same manner as oligomycin but the latter does not displace or compete with leucinostatin for its binding site (5, 6). Venturicidin is a glycoside and when it is present on the membrane the carbohydrate moiety extends into the region where leucinostatin binds, for the latter is displaced from this inhibitory site and can now perform its second function, namely, to uncouple, probably by transferring protons across the mitochondrial membrane. There are thus four different sites for binding antibiotic inhibitors of oxidative phosphorylation.

Effects of Nucleotide Analogs on Exchange Reactions

The introduction of methylene-bridged analogs of nucleoside di- and triphosphates by Myers and co-workers (7) and of imino-bridged analogs by Yount and his students (8, 9) has provided highly useful tools for examining many different kinases, phosphatases, and reactions related to nucleotide synthesis. Reports from several laboratories have documented the inhibition of mitochondrial ATPase by these analogs (10–14). We have used them for investigating the exchange reactions associated with oxidative phosphorylation with somewhat unexpected results (10). Here, for brevity, we will discuss only the effects of adenylylimidodiphosphate (AMP-PNP).

AMP-PNP is a competitive inhibitor of ATP hydrolysis by submitochondrial particles as well as by purified ATPase (Fig. 2 and reference 10). It inhibits the exchange of $^{32}P_i$ into ATP and of ^{18}O from water into P_i, but at 10 mM AMP-PNP there was no inhibition of ^{18}O exchange between water and ATP (10). Even at 10 mM, this analog does not inhibit oxidative phosphorylation nor does it alter the affinity for P_i of the oxidative phosphorylation system in submitochondrial particles.

Figure 3, which is taken from a recent paper by Cross and Boyer (15), presents a possible sequence of reactions that could account for the various exchanges as well as for the overall process of ATP synthesis. It would be logical to rationalize the failure of

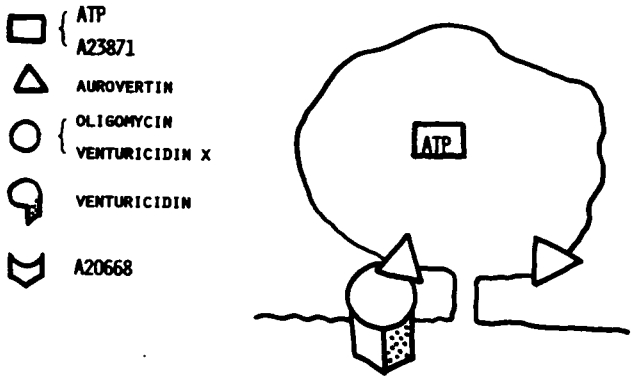


Fig. 1. Schematic localization, on mitochondrial inner membrane, of sites that bind inhibitory antibiotics. Antibiotic A20668 is probably identical with leucinostatin and A23871 has been named Efrapeptin in honor of a distinguished investigator of the ATPase. Venturicidin X is the aglycone of venturicidin.

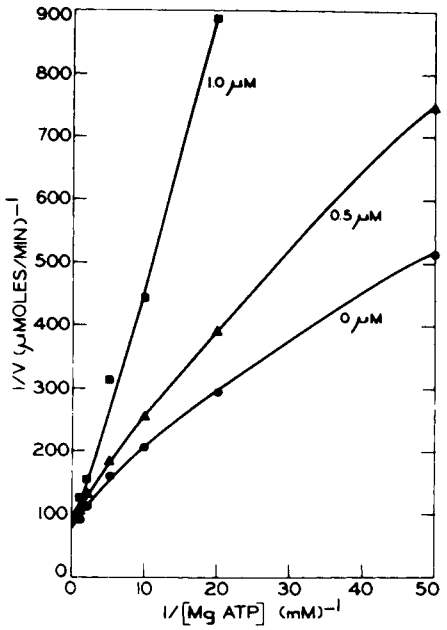
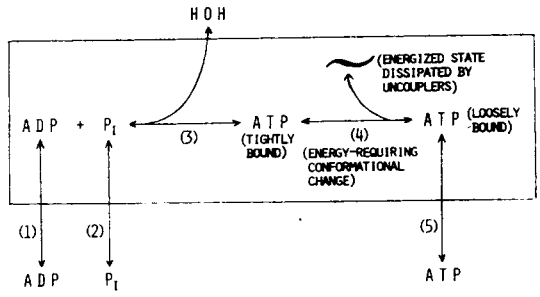


Fig. 2. Inhibition of ATPase in beef heart submitochondrial particles by AMP-PNP.

AMP-PNP to inhibit the ATP-water exchange by assuming that the analog blocks reaction 2, the dissociation of P_i from the enzyme after reaction 3 has occurred. It is somewhat difficult to rationalize that explanation with the failure of AMP-PNP to inhibit



STEPS REQUIRED FOR EXCHANGE REACTIONS

$P_i \rightleftharpoons HOH$	2, 3
$ATP \rightleftharpoons HOH$	3, 4, 5
$P_i \rightleftharpoons ATP$	2, 3, 4, 5

Fig. 3. A sequence for conformational coupling to ATP synthesis proposed by Cross and Boyer (15). Reproduced here with the kind of permission of the authors.

oxidative phosphorylation or to enhance it under conditions where the rate of ATP synthesis is limited by P_i concentration. These findings caused us to seek other information about the mechanism by which AMP-PNP inhibits so selectively. Before discussing that work, however, it is appropriate here to consider the sequence of the ATPase reaction.

With heart submitochondrial particles, adenosine PCP is a noncompetitive inhibitor of the ATP-$^{32}P_i$ exchange when $[ATP]/[P_i]$ is kept constant but their concentrations are varied. This exchange reaction is also inhibited by high concentrations of ATP and ADP when the ratio of these two nucleotides is kept constant. Furthermore, elevating the concentration of ITP and IDP, at constant ratio, inhibits the exchange of $^{32}P_i$ into ITP. All these conditions are indicative of trapping the enzyme in a central $E \cdot ADP \cdot P_i$ complex, and thus, the reaction is sequential with the order shown:

$$\begin{array}{ccccccc} & P_i & & ADP & & & ATP \\ & \Downarrow & & \Downarrow & & & \Downarrow \\ E & & E \cdot P_i & & E \cdot ADP \cdot P_i & E \cdot ATP & & E \end{array}$$

P_i leaves after ADP in the ATPase sequence and adds before ADP in the direction of oxidative phosphorylation.

The Kinetic Behavior of Purified ATPase (F_1)

In the standard ATPase assay, beef heart F_1 exhibits apparent negative cooperativity with ATP as the substrate just as does the rat liver enzyme (1). Increasing concentrations of a number of anions decrease the negative cooperativity and, at sufficiently high concentrations, provide linear plots and a considerably enhanced maximum velocity.

Hill coefficients of 0.5 are brought to approximately 1 by the anions. The anion activation requires a second protonated acid function(Br^- is an exception).For example, with malonate, it is the half-ionized species that is the activator. Negative cooperativity is not shown when ITP or GTP is the substrate and the extent of anion activation is much less than with ATP.

In the absence of activating anions, AMP-PNP changes the apparent negative cooperativity of purified mitochondrial ATPase to apparent positive cooperativity (16). As with submitochondrial particles (Fig. 2), inhibition of heart F_1 ATPase by AMP–PNP is competitive with ATP (K_i = 320 μM), for at infinite ATP concentration AMP-PNP has no effect on V_{max}.

With ITP as the substrate, AMP-PNP is an extremely effective noncompetitive inhibitor (Fig. 4) with a K_i of 8 nM. Similar effects are observed during GTP hydrolysis (16).

These data were interpreted to mean that AMP-PNP was acting, not at the catalytic site where ATP, ITP, and GTP bind when they are hydrolyzed, but at a regulatory site which probably binds ATP as well. This would account for the noncompetitive inhibition of ITP and GTP hydrolysis.

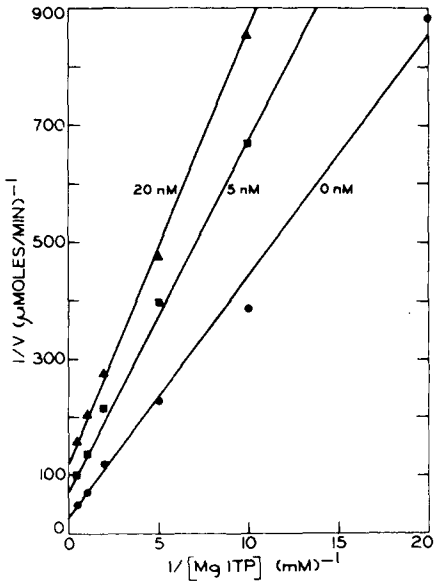


Fig. 4. Inhibition by AMP-PNP of ITP hydrolysis by purified beef heart mitochondrial ATPase. Note the concentration of AMP-PNP shown is nmolar.

To gain further information about the specificity of the proposed sites, the β-γ- imido analog of GTP (GMP-PNP) was tested as an inhibitor and it turned out to be a beautiful competitive inhibitor of ITP (Fig. 5) and GTP (16) hydrolysis. With ATP as substrate,

GMP-PNP is nearly competitive but there is substrate inhibition by high concentrations of ATP in the presence of the guanosine nucleotide analog (16).

GMP-PNP Inhibits Oxidative Phosphorylation

Although AMP-PNP has no effect on oxidative phosphorylation (12, 17), GMP-PNP is a powerful inhibitor. The data presented thus far indicate that GMP-PNP seeks the catalytic site on mitochondrial F_1. Since both hydrolysis and synthesis of ATP are inhibited by GMP–PNP, both of these processes may occur at the same site rather than at separate sites as has been proposed (12).

Chromium Complexes of Adenine Nucleotides as Inhibitors

Chromium ATP (18) competitively inhibits ATP hydrolysis by beef heart submitochondrial particles (Fig. 6) without abolishing the apparent negative cooperativity. Chromium ADP also inhibits competitively. In contrast, both chromium ATP (Fig. 7) and CrADP are noncompetitive inhibitors of ITP hydrolysis. Rat liver mitochondrial ATPase responds in the same qualitative manner to the chromium complexes of the adenine nucleotides as does the enzyme in submitochondrial particles. In the case of the purified ATPase it has been established that the effective inhibitor is the tridentate form of CrATP (19).

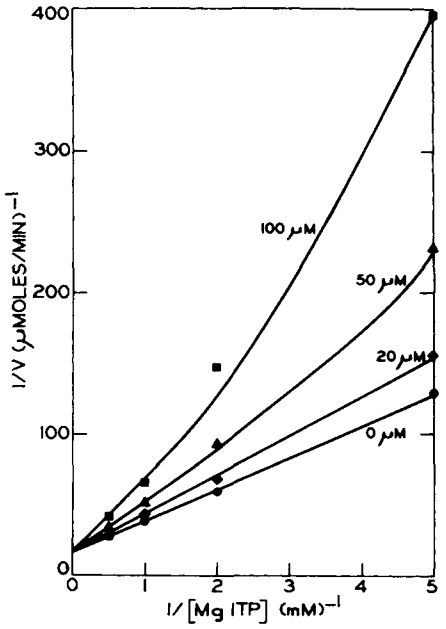


Fig. 5. Inhibition by GMP-PNP of ITP hydrolysis by purified beef heart mitochondrial ATPase.

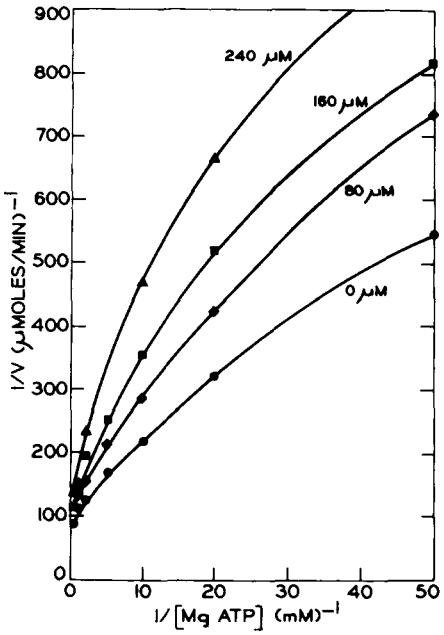


Fig. 6. Effect of the chromium complex of ATP on the hydrolysis of ATP by beef heart submitochondrial particles.

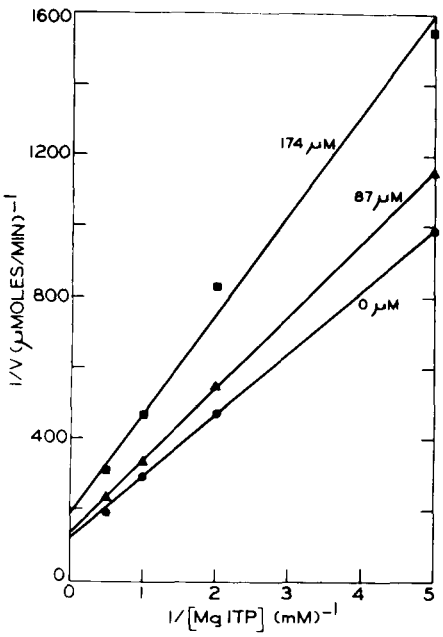


Fig. 7. Effect of the chromium complex of ATP on hydrolysis of ITP by beef heart submitochondrial particles.

DISCUSSION

These results lead to conclusions that are summarized in Fig. 8. We postulate the presence of two distinct types of binding sites on mitochondrial ATPase. A catalytic site (C) binds ATP, ITP, or GTP as substrates and also binds the inhibitory GMP-PNP. The effect of the latter is therefore competitive inhibition of the hydrolysis of ITP and GTP, nearly competitive inhibition of ATP hydrolysis, and inhibition of ATP synthesis in oxidative phosphorylation.

Because AMP-PNP inhibits the hydrolysis of ITP and GTP in a noncompetitive manner, we assume it combines at the regulatory site (R).ATP inhibits its own hydrolysis by binding at the regulatory site to cause the apparent negative cooperativity and to suppress V_{max} of ATP hydrolysis.

ADP is also an inhibitor at the R site and the relative effectiveness of the inhibition is designated in Fig. 8 by the thickness of the minus sign. Competition between ATP and AMP-PNP for binding at the R site is evidenced by the fact that K_i is approximately 50 times higher when ATP is the substrate as compared with GTP or ITP. Binding of GTP and ITP at the R site must therefore be very weak. AMP-PNP binding at the R site does not affect oxidative phosphorylation.

Chromium complexes of ATP and ADP appear to exert their inhibitory effect, at least in part, at the R site for they are noncompetitive vs. GTP and ITP and competitive

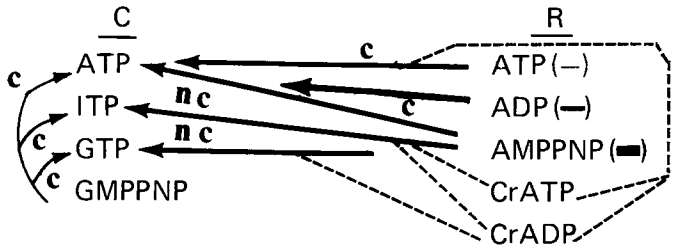


Fig. 8. Nucleotide binding sites on ATPase. C designates competitive inhibition and NC, noncompetitive.

vs. ATP. It is important to understand that at infinite concentration of ATP the chromium complex would be displaced from the R site and V_{max} would represent the velocity achievable while ATP exerted its inhibitory effect at the R site.

It appears that part of the influence exerted by activating anions is the result of their diminishing the effectiveness of ATP at the R site. They abolish the negative cooperativity and they increase the velocity of ATP hydrolysis much more than that of GTP or ITP. Aurovertin eliminates the enhancement by bicarbonate of ATPase activity (20).

We have no evidence yet as to the number of C and R sites per molecule of ATPase. Nor do we know whether C and R sites are interconvertible. Current work is aimed at obtaining answers to these questions as well as to obtaining further information about the mechanism of the synthesis of ATP. The results of kinetic experiments summarized here provide no evidence that separate sites are involved in ATP synthesis and hydrolysis.

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