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Beef heart mitochondrial F_1-ATPase: inhibition by azidoadenyl-5′-yl imidodiphosphates and cooperative binding of substrate

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Two ATP analogs, 2- and 8-azidoadenyl-5′-yl imidodiphosphate, were synthesized, purified and utilized as inhibitors of soluble beef heart mitochondrial F_1-ATPase under non-photolytical conditions. In the range of 5 μM to 3 mM ATP, the initial rates of ATP hydrolysis in the presence and absence of the inhibiting ATP analogs can be adequately described by two pairs of K_m and V_{max} values (3 μM, 8.5 μmol ATP/min per mg; 255 μM, 42.0 μmol ATP/min per mg). With increasing inhibitor concentrations, the apparent $K_{m,2}$ increases as in competitive inhibition, while $V_{max,1}$ decreases as in non-competitive inhibition. The K_i values derived for both types of inhibition are similar, but strongly different for 2- and 8-azido-AMP-PNP (4 μM and 460 μM, respectively). The decrease of the high-affinity V_{max} is compensated by an increase in low-affinity catalysis, resulting in a constant sum of maximal velocities. These data can be described by a model where two sites interact with negative cooperativity in binding of substrate.

Introduction

Mitochondrial ATP synthase is composed of a membrane-bound, proton-conducting complex (F_0) and a catalytic segment (F_1) capable of promoting ATP hydrolysis after detachment from the membrane. F_1 is composed of five unequal subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ [1–3]. Catalysis is associated with the β subunit, although the question about the number of catalytically-active β subunits is not resolved [4]. In the binding-change mechanism of ATP synthesis proposed by Boyer [5], all β subunits are assumed to be catalytic and to interact with each other. Based on data on subunit scrambling [6], anion activation [7], hysteretic inhibition [8] and covalent modifications [9–11], other authors have concluded that less than three catalytic sites interacting with substrate binding sites are present.

Experimentally, strong cooperativity has been observed in the transition from uni-site to bi- [12,13] or multi-site catalysis [14]. Cooperative phenomena in the ATP concentration range where bi- and tri-site catalysis might occur (0.05–5000 μM) are less well documented. For kinetic analyses of F_1 activity in this range of substrate concentrations, at least two K_m values were necessary [14–17], and Hill coefficients below unity have been observed [18–20]. However, it has been realized earlier [18] that this type of kinetics may be due to either the presence of two independent active sites with different kinetic parameters, negative cooperativity between two identical catalytic sites, or interactions between a catalytic and a modifying substrate binding site. It was also pointed out that a distinction between these alternatives cannot be made on the basis of rate measurements as a function of substrate concentrations [21,22]. The possibility that such a distinction might be feasible in the presence of inhibitors was apparently not systematically explored.

In the present study, we describe the use of the inhibitors 2- and 8-azido-AMP-PNP as inhibiting ATP analogs under non-photolytic conditions. We show that in the micro- to millimolar ATP concentration range and in the absence and presence of inhibiting AMP-PNP derivatives, the ATPase activity of F_1 can be adequately represented assuming two substrate binding sites, and that it is possible to distinguish between

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Abbreviation: AMP-PNP, adenyl-5′-yl imidodiphosphate.

negative cooperativity and independent, but unequal catalytic sites with the use of the azido-AMP-PNP isomers as reversible inhibitors.

Materials and Methods

Commercial AMP-PNP (Boehringer-Mannheim) was freed from contaminants (mainly AMP-PN) by isocratic anion-exchange chromatography on Dowex 1X2 (200–400 mesh, 1.4×28 cm) using 0.3 M NaCl in 20% methanol (v/v) as the eluent.

8-Bromo-AMP and 8-azido-AMP were prepared essentially according to Czarnecki et al. [23]. 8-Azido-AMP was coupled to imidodiphosphoric acid (PNP) as described by Yount et al. [24]. The purification of 8-azido-AMP-PNP and 8-azido-ATP by anion-exchange chromatography on DEAE-cellulose was monitored in pertinent fractions by HPLC analysis and determination of the acid-labile phosphate to 8-azido-adenine ratio. While 8-azido-AMP-PNP tends to lose the γ-phosphate group in the elution buffer, it is stable in absolute methanol for at least two months at -20°C. The structure of 8-azido-AMP-PNP, 8-azido-AMP and 8-bromo-AMP was confirmed by fast-ion-bombardment mass spectrometry (Dr. D. Müller and D. Grzelak, Analytical Chemistry Division, Ruhr-University Bochum). The microsynthesis of tritiated 8-azido-AMP-PNP has been reported by Boos et al. [25].

2-Azidoadenosine was prepared from 2-chloroadenosine as described [26] and converted to 2-azido-AMP according to Cusack and Born [27]. Coupling of 2-azido-AMP with imidodiphosphate and purification of 2-azido-AMP-PNP was done as described above for 8-azido-AMP-PNP. The structure of 2-azido-AMP-PNP was confirmed by time-of-flight (TOF) mass spectrometry. Like 2-azido-ATP, 2-azido-AMP-PNP occurs in spectrally distinct tetrazole and azido forms. Within the limits of accuracy, the isomerization rates and the spectral characteristics of 2-azido-AMP-PNP are the same as those reported for 2-azido-ATP [28].

Inorganic phosphate liberated from adenine nucleotide derivatives by acid hydrolysis [24] was quantitated as described [29]. The concentration of the adenine, 8-azido-adenine and 2-azido-adenine moieties was determined spectroscopically at 260, 281 and 270 nm using the extinction coefficients 15 400, 13 300 [30] and 15 500 [28], respectively. Purified AMP-PNP, 2- and 8-azido-AMP-PNP showed phosphate/adenine ratios of 1.91 ± 0.17, 2.08 ± 0.07 and 2.07 ± 0.31, respectively.

Bovine mitochondrial F_1-ATPase was prepared according to Penefsky [31]. Before use, F_1-ATPase was desalted and freed from loosely bound nucleotides by the centrifuged-column method [32] in the presence of EDTA and ATP. Spectrophotometric determination of ATPase activity was done according to Pullman et al. [33] at 30°C in the presence of $MgCl_2$ (5 mM), MgATP (2.5 mM), Na-phospho*enol*pyruvate (2 mM), NADH (0.25 mM), pyruvate kinase (4 U/ml) and lactate dehydrogenase (11 U/ml) in 0.25 M sucrose containing 50 mM Tris-HCl (pH 8). The reaction was started by the addition of F_1 (0.5–0.6 μg/ml) and initial rates were measured within 10 to 30 s. As observed before with AMP-PNP [34,35], the inhibition by 2- and 8-azido-AMP-PNP increased in time, and more rapidly so at low ATP concentrations. In the presence of inhibitors therefore, the initial rates at ATP concentrations below 16 μM are probably underestimates, and consequently the $K_{m,1}$ values in Table I represent upper limits. Solutions containing F_1-ATPase and the inhibitors 2- and 8-azido-AMP-PNP were protected from light to prevent photoaffinity labeling and maintain equilibrium conditions. The inhibition by AMP-PNP derivatives can be reversed by the centrifuged-column method using Sephadex G-50 [32].

Data analysis

Initial rates of ATP hydrolysis by F_1 as a function of substrate concentration were fitted by a non-linear regression procedure to an equation describing two independent catalytic sites or, as shown below, two

TABLE I

Inhibition of F_1-catalyzed ATP hydrolysis by 2- and 8-azido-AMP-PNP

Kinetic data analyzed on the basis of Eqn. 6 as described in Materials and Methods. The $K_{m,1}$ values are upper limits; see Materials and Methods. [a] $V_{max,1} + V_{max,2}$.

	2-N_3-AMP-PN (μM)				8-N_3-AMP-PN (μM)			
	0	3.7	8.3	16.6	0	68	136	340
$K_{m,1}$ (μM)	4.4± 1.4	0.0± 0.7	3.5± 2.1	3.0± 1.2	1.4± 0.5	0.6± 0.7	0.8± 0.8	1.2± 1.4
$V_{max,1}$ (μmol/min per mg)	8.9± 1.3	3.0± 0.4	2.0± 0.4	1.4± 0.2	8.0± 0.6	4.8± 0.6	5.9± 0.7	4.2± 0.9
$K_{m,2}$ (μM)	210 ±30	290 ±20	650 ±80	1020 ±100	300 ±30	280 ±30	370 ±50	530 ±100
$V_{max,2}$ (μmol/min per mg)	42.2± 1.2	47.2± 1.0	52.7± 3.0	51.5± 2.7	41.8± 1.2	45.3± 1.6	45.6± 2.1	51.3± 3.8
ΣV_{max} [a]	51.1± 1.2	50.2± 1.0	54.7± 3.0	52.9± 2.7	49.8± 1.1	50.1± 1.5	51.5± 2.0	55.5± 2.7

sites interacting with strong negative cooperativity (Eqn. 6). This equation was derived on the basis of a model assuming two substrate binding sites as shown in Scheme I. This scheme contains the molecular species expected to be present in a dimeric enzyme [36] and represents a simple sequential interaction model suitable for the description of positive or negative cooperativity in both binding and catalysis.

$$[E]+[S] \overset{K_{s,1}}{\Longleftrightarrow} [ES] \xrightarrow{k_2} [P]$$

$$[ES]+[S] \overset{K_{s,2}}{\Longleftrightarrow} [ES_2] \xrightarrow{k_{22}} [P]$$

$$[EI]+[S] \overset{K_{s,3}}{\Longleftrightarrow} [EIS] \xrightarrow{\alpha k_2} [P]$$

$$[E]+[I] \overset{K_{i,1}}{\Longleftrightarrow} [EI]$$

$$[EI]+[I] \overset{K_{i,2}}{\Longleftrightarrow} [EI_2]$$

Scheme I. Catalysis and ligand binding at an enzyme with two initially identical binding sites.

With $V_{max} = k_{22}E_{tot}$, $i_1 = [I]/K_{i,1}$, and $i_2 = [I]/K_{i,2}$, Eqn. 1 is obtained:

$$v/V_{max} = \frac{\frac{k_2}{k_{22}}K_{s,2}\left[1+\alpha i_1\frac{K_{s,1}}{K_{s,3}}\right][S]+[S]^2}{K_{s,1}K_{s,2}(1+i_1+i_1i_2)+K_{s,2}\left[1+i_1\frac{K_{s,1}}{K_{s,3}}\right][S]+[S]^2} \quad (1)$$

This equation can be transformed into two linear hyperbolas of the Michaelis-Menten type as shown in Eqn. 2.

$$v/V_{max} = \frac{a[S]+[S]^2}{b+c[S]+[S]^2} = \frac{\left[\frac{1}{2}-\frac{c/2-a}{c\sqrt{1-4b/c^2}}\right][S]}{\frac{c}{2}\left[1-\sqrt{1-4b/c^2}\right]+[S]} + \frac{\left[\frac{1}{2}+\frac{c/2-a}{c\sqrt{1-4b/c^2}}\right][S]}{\frac{c}{2}\left[1+\sqrt{1-4b/c^2}\right]+[S]} \quad (2)$$

with

$$a = \frac{k_2}{k_{22}}K_{s,2}\left[1+\alpha i_1\frac{K_{s,1}}{K_{s,3}}\right]$$

$$b = K_{s,1}K_{s,2}(1+i_1+i_1i_2) \quad \text{and}$$

$$c = K_{s,2}\left[1+i_1\frac{K_{s,1}}{K_{s,3}}\right]$$

In the absence of cooperativity involving the inhibitor (system B2b in Ref. 37), the following relationships apply: $K_{i,2} = 4K_{i,1} = 2K_i$, $i_1 = 2i$, $i_2 = i/2$, $i_1i_2 = i^2$ and $K_{s,3} = 2K_{s,1}$.Introducing the cooperativity factor $n = 4K_{s,1}/K_{s,2}$ and defining $i = [I]/K_i$, Eqn. 2 can therefore be simplified to yield Eqn. 3:

$$v/V_{max} = \frac{\left[\frac{\sqrt{1-n}-1}{2\sqrt{1-n}}+\frac{k_2(1+\alpha i)}{k_{22}(1+i)\sqrt{1-n}}\right][S]}{\frac{K_{s,2}}{2}(1+i)(1-\sqrt{1-n})+[S]} + \frac{\left[\frac{\sqrt{1-n}+1}{2\sqrt{1-n}}-\frac{k_2(1+\alpha i)}{k_{22}(1+i)\sqrt{1-n}}\right][S]}{\frac{K_{s,2}}{2}(1+i)(1+\sqrt{1-n})+[S]} \quad (3)$$

Because of the square root terms, Eqn. 3 is applicable only for values of $n < 1$, i.e., for negative cooperativity in the binding of substrate. With $\sqrt{1-n}$ approx. $1-n/2$, Eqn. 3 is approximated by Eqn. 4:

$$v/V_{max} = \frac{\left[\frac{k_2(1+\alpha i)}{k_{22}(1+i)(1-n/2)}-\frac{n}{4-2n}\right][S]}{K_{s,1}(1+i)+[S]} + \frac{\left[1-\frac{k_2(1+\alpha i)}{k_{22}(1+i)(1-n/2)}+\frac{n}{4-2n}\right][S]}{K_{s,2}(1+i)(1-n/4)+[S]} \quad (4)$$

For $n \ll 1$, the approximation $1-n/2 \approx 1$ is justified and Eqn. 5 is obtained:

$$v/V_{max} = \frac{\left[\frac{k_2(1+\alpha i)}{k_{22}(1+i)}-\frac{n}{4}\right][S]}{K_{s,1}(1+i)+[S]} + \frac{\left[1-\frac{k_2(1+\alpha i)}{k_{22}(1+i)}+\frac{n}{4}\right][S]}{K_{s,2}(1+i)+[S]} \quad (5)$$

In cases with strong negative cooperativity in binding and moderate positive catalytic cooperativity, $n/4 \ll k_2(1+\alpha i)/k_{22}(1+i)$ applies and Eqn. 6 is obtained, where the substrate dissociation constants are replaced by Michaelis-Menten constants:

$$v/V_{max} = \frac{(V_{max,1}/V_{max})[S]}{K_{M,1}(1+i)+[S]} + \frac{(V_{max,2}/V_{max})[S]}{K_{M,2}(1+i)+[S]} \quad (6)$$

with

$$V_{max,1}/V_{max} = \left[\frac{k_2(1+\alpha i)}{k_{22}(1+i)}\right]$$

$$V_{max,2}/V_{max} = \left[1-\frac{k_2(1+\alpha i)}{k_{22}(1+i)}\right]$$

In this equation, the apparent K_m values are linear functions of the inhibitor concentration, just as in the

competitive inhibition of two independent catalytic sites characterized by $K_{m,1}$ and $K_{m,2}$. In contrast to simple competitive inhibition, however, $V_{max,1}$ in Eqn. 6 decreases with increasing inhibitor concentrations (for $\alpha < 1$), while the sum of $V_{max,1}$ and $V_{max,2}$ remains constant. If the turnover of a substrate molecule is completely abolished by the presence of the inhibitor at the second binding site ($\alpha = 0$), $V_{max,1}$ is affected as in a simple non-competitive inhibition, and the values of K_i determined from plots of K_m or $1/V_{max,1}$ vs. [I] are expected to be the same. For $1 > \alpha > 0$, the K_i values deduced from $V_{max,1}$ data are larger than those from K_m values. For $\alpha = 1$, $V_{max,1}$ and $V_{max,2}$ would remain constant, and on the basis of Eqn. 6, it would not possible to distinguish the model shown in Scheme I from competitive inhibition at two independent, unequal catalytic sites. Finally, if the inhibitor is assumed to exert negative cooperativity on its own binding (cooperativity factor $m = 4K_{i,1}/K_{i,2}$), but not on that of the substrate, the apparent $K_{m,1}$ in Eqn. 6 changes from $K_{m,1}(1 + i)$ to $K_{m,1}\{1 + i(im + 1)/(i + 1)\}$ for small values of m. This has the effect of the apparent $K_{m,1}$ changing to a lesser extent than $K_{m,2}$ at increasing inhibitor concentrations.

Results

The ATPase activity of beef heart mitochondrial ATPase has been widely reported not to obey simple Michaelis-Menten kinetics, giving rise to non-linear Lineweaver-Burk or Eadie-Hofstee plots [14–20]. As shown in Fig. 1, this is also the case in the presence of increasing concentrations of the inhibitors 2- and 8-azido-AMP-PNP. The curvature of the Eadie-Hofstee plots is typical for either negative cooperativity or the presence of at least two independent and unequal binding sites [22,36]. In the present work, two pairs of

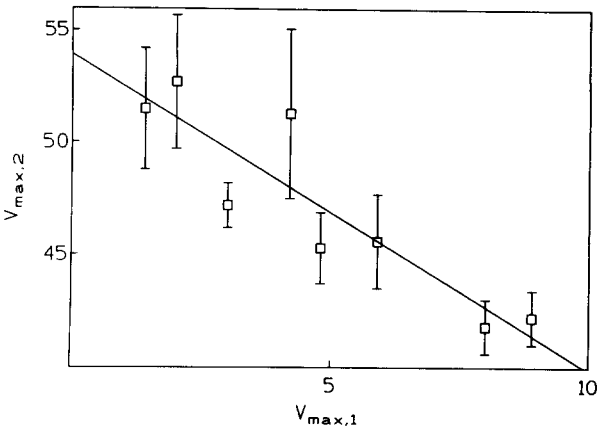

Fig. 2. Correlation of the maximal velocities of the apparent low and high-affinity catalytic sites of F_1, $V_{max,2}$ and $V_{max,1}$, respectively. Data of Table I. Slope, -1.41 ± 0.28; intercept, 53.9 ± 1.5; r^2, 0.80.

K_m and V_{max} values were sufficient for an adequate fit of the data (Table I). The average K_m and V_{max} values in the absence of inhibitors (3 and 255 μM, 8.5 and 42.0 μmol ATP/min per mg) are in the same range as those reported by Gresser et al. [15] for F_1-ATPase and Muneyuki and Hirata [38] for submitochondrial particles. In the presence of increasing inhibitor concentrations, it is seen that $K_{m,2}$ and $V_{max,2}$ increase, while the values of $V_{max,1}$ actually decrease. Remarkably, the sum of $V_{max,1}$ and $V_{max,2}$ appears to stay constant (52.0 ± 2.2 μmol ATP/min per mg), and an approximately linear relationship is found between these parameters (Fig. 2). Plots of $1/V_{max,1}$ and $K_{m,2}$ vs. inhibitor concentration are linear (Figs. 3 and 4).

Changes of $V_{max,1}$ and $V_{max,2}$ brought about by inhibiting substrate analogs are not readily expected from an enzyme with two independent and unequal binding sites. However, a model (Scheme I) based on two sites interacting with negative cooperativity predicts this type

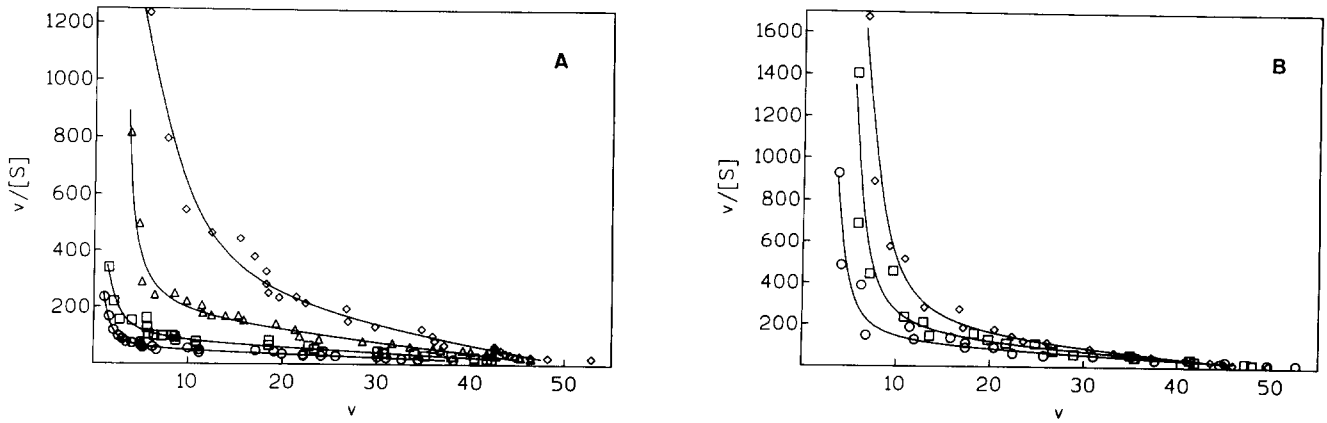

Fig. 1. Eadie-Hofstee plots of the inhibition of ATP hydrolysis by 2- and 8-azido-AMP-PNP. Initial rates of ATPase activity of F_1 were determined in the presence of (A) 2-azido-AMP-PNP (◇, 0 μM; △, 3.7 μM; □, 8.3 μM; ○, 16.6 μM) and (B) 8-azido-AMP-PNP (◇, 0 μM; □, 136 μM; ○, 340 μM). Reactions were started by addition of the enzyme. For other details, see Materials and Methods. The curves were calculated using Eqn. 6 with the apparent V_{max} and K_m values from Table I.

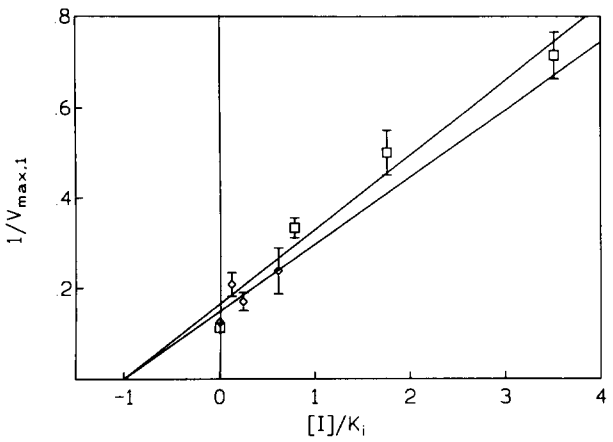

Fig. 3. Apparent non-competitive inhibition by azido-AMP-PNP. $1/V_{max,1}$ as a function of the inhibitor concentration; □, 2-azido-AMP-PNP, $K_i = 4.7$ μM; ◇, 8-azido-AMP-PNP, $K_i = 553$ μM. Data of Table I.

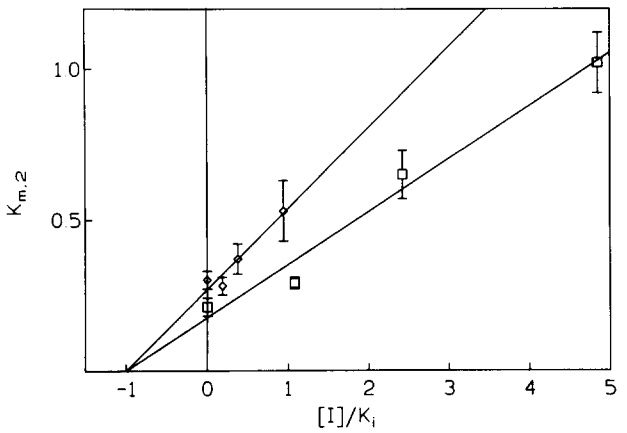

Fig. 4. Apparent competitive inhibition by azido-AMP-PNP. $K_{m,2}$ as a function of the inhibitor concentration; □, 2-azido-AMP-PNP, $K_i = 3.4$ μM; ◇, 8-azido-AMP-PNP, $K_i = 360$ μM. Data of Table I.

of data (Table II). Thus, in the presence of competitive inhibitors, negative cooperativity becomes distinguishable from a model with two independent, unequal sites.

TABLE II

Inhibition characteristics in models with negative cooperativity and with independent catalytic sites

2 sites, negative cooperativity [a]	2 independent, unequal sites
$V'_{max,1} = \frac{V_{max,1}}{1+[I]/K_i}$	$V_{max,1}$ = constant
$V'_{max,2} = \frac{V_{max,2}}{1+[I]/K_i}$	$V_{max,2}$ = constant
$K'_{m,1} = K_{m,1}(1+[I]/K_i)$	$K'_{m,1} = K_{m,1}(1+[I]/K_i)$
$K'_{m,2} = K_{m,2}(1+[I]/K_i)$	$K'_{m,2} = K_{m,2}(1+[I]/K_i)$

[a] Based on Eqn. 6.

Because the values of $K_{m,1}$ represent upper limits (see Materials and Methods), the interaction factor for the negative cooperativity in substrate binding ($K_{m,2}/4K_{m,1}$) can only be estimated (> 20). The positive catalytic cooperativity ($k_{22}/2k_2$) is modest (3.2) but significant. Both effects are much smaller than those observed in the transition from subnanomolar to micro- and millimolar concentrations of free ATP [12,14].

Discussion

In the concentration range of 5 μM to 3 mM ATP, the ATPase activity of F_1 can be described by two pairs of K_m and V_{max} values (Table I). Similar results have been reported by others [14,15,17,39]. Sometimes, three K_m values were necessary for adequate fitting of steady-state ATPase activities [16,17]. These differences may be due to variations in the treatment of F_1 [17], or to heterogeneity in the enzyme preparations. The alternate site mechanism proposed by Boyer is based on three interacting catalytic sites [5,15,40], predicts negative cooperativity in substrate binding, and might therefore be expected to involve three K_m values. However, two pairs of K_m and V_{max} values were sufficient to reproduce experimental ATPase activity in a wide range (0.05 μM to 2 mM) of ATP concentrations [15]. Similar results were obtained using data calculated with the complete set of rate constants [15] from 0.5 μM to 2.35 mM ATP (not shown).

Whether the two sets of K_m and V_{max} values determined in the absence of inhibitors (Table I) reflect two independent catalytic sites with different affinity or two substrate binding sites interacting in negative cooperativity is a question that cannot be answered on the basis of ATPase measurements alone, because the corresponding rate equations are equivalent [22] (see Materials and Methods). Earlier experimental evidence against independent sites was based on photoaffinity inactivation data with 8-azido-ATP [39]. The inhibition characteristics reported in the present work, i.e., apparent noncompetitive inhibition in $V_{max,1}$ (Fig. 3), and the increase in $V_{max,2}$ at the expense of $V_{max,1}$ (Fig. 2) are not expected from an enzyme containing independent catalytic sites (Table II). These data, together with an apparent competitive inhibition in $K_{m,2}$ (Fig. 4), are, however, in accord with a mechanism involving two identical binding sites, negative cooperativity in substrate binding and positive catalytic cooperativity (Scheme I).

This mechanism is probably not the only one capable of rationalizing the inhibition data presented in this communication. There are similarities in some respects to the model put forward by Recktenwald and Hess [7] who assumed negative cooperativity between two unequal substrate binding sites, only one of which is

catalytic. When experimental data obtained with yeast F_1 were fitted to this model, the substrate binding sites turned out to have about the same affinities (4.2 μM and 4 μM, respectively) for the catalytic and regulatory site [7]. If this were true also for the affinities of competitive inhibitors such as azido-AMP-PNP, the velocity equation for this model would become indistinguishable from that for Scheme I (not shown). A variety of other observations point to mechanisms involving interacting catalytic and noncatalytic substrate binding sites [4,6,8–11,41–43].

It is apparent from Scheme I that ATPase measurements alone or in the presence of inhibitors cannot distinguish between catalytically active and inactive substrate binding sites engaged in cooperative interactions. Therefore, the conclusion that can be derived from the present work is that two substrate binding sites, one or both of them catalytically active, are involved in ATP hydrolysis occurring in the micromolar to millimolar range of ATP concentrations, and that the absence of Michaelis-Menten kinetics in this range is due to negative cooperativity in substrate binding and positive cooperativity in catalysis. If one includes the high-affinity ATP binding site involved in uni-site catalysis [12,14], there appear to be three substrate binding sites on F_1-ATPase, all of them interacting with negative cooperativity.

The inhibitors, 2- and 8-azido-AMP-PNP used under non-photolytic conditions, differ in their inhibitory potency by two orders of magnitude (Table I). This is probably due to different orientations of the purine ring relative to the ribose. In purine nucleosides with bulky substituents in position 8, the conformation is largely restricted to syn [44,45], while both syn and anti conformations are possible with ATP [46]. For steric reasons, 2-substituted purine nucleosides are likely to prefer the anti conformation. Therefore, the superior inhibitory potency of 2-azido-AMP-PNP, like the strong binding affinities of 2-azido-ATP and 2-azido-ADP [5,28,47,48], may be due to a preference of F_1 for nucleotides in the anti conformation. Interestingly, yeast hexokinase appears to prefer nucleotides in the syn conformation, as it is inhibited by 8-azido-AMP-PNP to a much higher degree than by the 2-substituted isomer [49].

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