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Inhibitory effect of NaN_3 on the F_0F_1 ATPase of submitochondrial particles as related to nucleotide binding

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The inhibitory effects of NaN_3 on the F_0F_1 ATPase of beef heart submitochondrial particles were investigated. It was shown that NaN_3 inhibited the ATPase activity only in the presence of ATP or ADP and the inhibition proceeded slowly. Analysis of the time-course of the inhibition process lead to a conclusion that an ATP binding site which has an apparent K_d of 14.0 ± 8.7 μM is responsible for the increase of NaN_3 sensitivity. This value agreed well with the low K_m of ATP hydrolysis characterized before (Muneyuki, E., and Hirata, H. (1988) FEBS Lett. 234, 455–458) and in the range of so-called bi-site catalysis. The same conclusion was derived as for isolated F_1 ATPase. From similar analysis,the K_d of this site for ADP was deduced to be 1.34 ± 0.45 μM, which also agreed with that reported by Pedersen (Pedersen, P.L. (1975) Biochem. Biophys. Res. Commun. 64, 610–616) and also in the same range as reported for the low K_m of ATP synthesis by activated submitochondrial particles. These results suggest that hydrolysis through the low K_m mode of ATPase reaction leads the enzyme NaN_3 sensitive form and this reaction cycle corresponds to the low K_m mode of ATP synthesis.

Introduction

Membrane-bound proton translocating ATPase (F_0F_1 ATPase) has a central role in energy transduction by converting the protonic electrochemical potential to the chemical potential of ATP [1]. In these 30 years, the mechanism of ATP hydrolysis has been intensively studied using F_1 portion and many details have become known [2–6]. One of the most prominent characteristics of this enzyme is the apparent negative cooperativity of ATP hydrolysis [7]. Under steady-state conditions, non-linear fitting analyses revealed two apparent K_m values of 1–10 μM and 100–300 μM [1,8–10]. Sometimes, a third K_m of around or above 1 mM has been reported [1,8,10]. The V_{max} corresponding to the K_m of 1–10 μM is usually in the range of 1–10 μmol mg^{-1} min^{-1} and the V_{max} corresponding to higher K_m's are about ten fold higher than the former. In addition, under a single-turnover conditions, a catalytic site with very high affinity ($K_d = 10^{-12}$ M) and very low turnover rate (10^{-4} s^{-1}) has been reported for mitochondrial F_1 ATPase [11,12,3]. The features of this single turnover are well consistent with the binding change mechanism proposed by Boyer's group [2,9,13] and these reactions with different K_d or K_m values are called uni-site, bi-site and tri-site catalysis, respectively [14]. Previously, we reported that both the ATPase reaction and proton translocation exhibited negative cooperativity characterized with two apparent K_m values of about 10 μM and 200 μM using submitochondrial particles (SMP) [15]. However, characteristics of these kinetic modes with different K_m values were not clear. In order to get insights into the nature of these two kinetic modes, it might be useful to characterize inhibitor sensitivity. Among many inhibitors which act to F_0F_1 ATPase, Vasilyeva et al. reported that NaN_3 acts on the ATPase of AS particles only in the presence of ATP [16]. On the other hand, Noumi et al. reported that uni-site catalysis of F_1 ATPase from *E. coli* was not inhibited by NaN_3 [17]. These results mean that ATP binding to some catalytic or non-catalytic site other than the uni-site of the F_0F_1

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Abbreviations: EF_1, F_1-ATPase from *Escherichia coli*; SMP, submitochondrial particles.

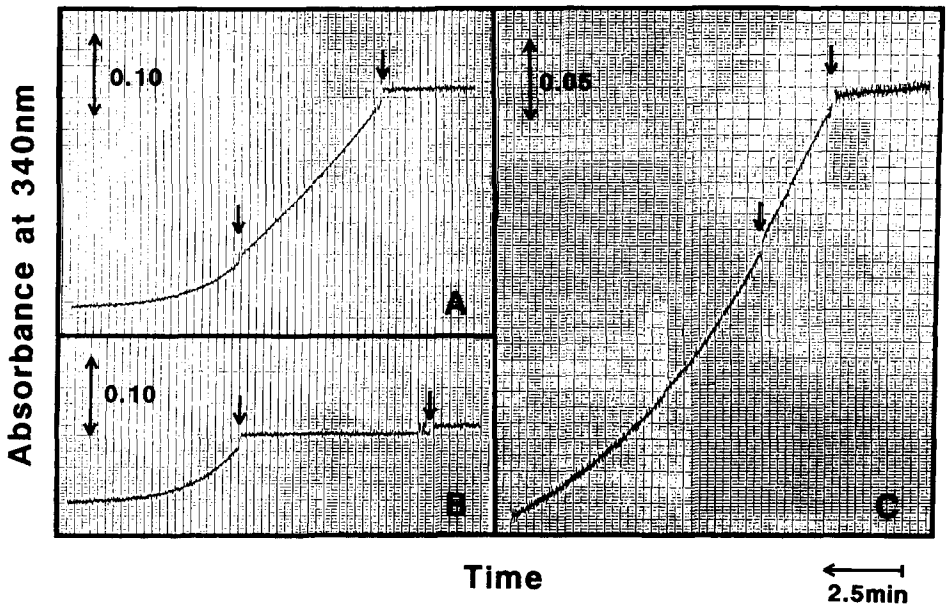

Fig. 1. Effect of NaN_3 on ATPase activity of submitochondrial particles. ATPase activity was measured spectrophotometrically as described in Materials and Methods. (A) The reaction was started by the addition of ATP (400 μM) as indicated by the right arrow and NaN_3 (500 μM) was added subsequently as indicated by the left arrow. (B) The same as in (A), except the order of addition was reversed. NaN_3 (500 μM) was added first at the right arrow and ATP (400 μM) was added subsequently as indicated by the left arrow. (C) The same as in (A), except the ATP concentration was lowered to 8 μM. The amount of SMP was increased so that the activity was measured more precisely.

ATPase greatly enhances NaN_3 sensitivity. Here we analyzed the inhibitory effect of NaN_3 as a function of ATP concentration and the results indicated that binding of ATP to a site which has an apparent K_d of 14.0 ± 8.7 μM increases NaN_3 sensitivity. * The apparent K_d for ATP is close to the low K_m characterized before [15] corresponding to that of so-called bi-site catalysis. The same conclusion was derived for isolated F_1 ATPase. It was also shown that NaN_3 sensitivity was increased by binding of ADP and an apparent K_d for ADP was deduced to be 1.34 ± 0.45 μM and partially reduced by inorganic phosphate.

Materials and Methods

Reagents

Pyruvate kinase was purchased from Sigma (type II, P-1506). Lactate dehydrogenase was purchased from Boehringer (127 876). Other reagents were of the highest grade commercially available.

Preparation of submitochondrial particles and F_1 ATPase

SMP were prepared from beef heart mitochondria suspended in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, by sonication followed by centrifugation [18]. Typically, specific activity for ATP hydrolysis at 4 mM of ATP was between 2 and 3 μmol mg^{-1} min^{-1} (at 25°C) and oligomycin sensitivity was more than 95%. F_1 ATPase was isolated by the chloroform treatment method by Linnet et al. [19] and further purified by DEAE-Toyopearl ion-exchange chromatography. The specific activity of the purified enzyme was between 50 and 60 μmol mg^{-1} min^{-1}.

Measurement of ATPase activity

ATPase activity was measured spectrophotometrically using ATP regenerating system [20]. The assay mixture contained 50 mM Tris-SO_4 (pH 8.0), 2 mM $MgSO_4$, 7 mM KCN, 2.5–5 mM phospho*enol*pyruvate, 0.3 μg/ml FCCP, 34 μg/ml pyruvate kinase, 45 μg/ml lactate dehydrogenase, 0.2 mM NADH, and indicated amount of ATP and NaN_3. As for F_1 ATPase, FCCP and KCN were omitted. Coupling enzymes were used without desalting.

When necessary, ATP solution was pre-incubated with the assay mixture and contaminating ADP was completely converted to ATP before assay.

Rate constants (τ) of inhibition process in the presence of ATP or ADP were deduced by measuring the

* In the present study, it is assumed that the enzyme fraction which binds ATP under steady-state hydrolysis conditions is sensitive to NaN_3. On this assumption, the K_d obtained in this study should not be regarded as the real K_d which reflects the first binding step of enzyme reaction and it should be rather close to the apparent K_m of ATPase reaction. However, for simplicity, we use the term 'K_d' or 'apparent affinity' throughout this article.

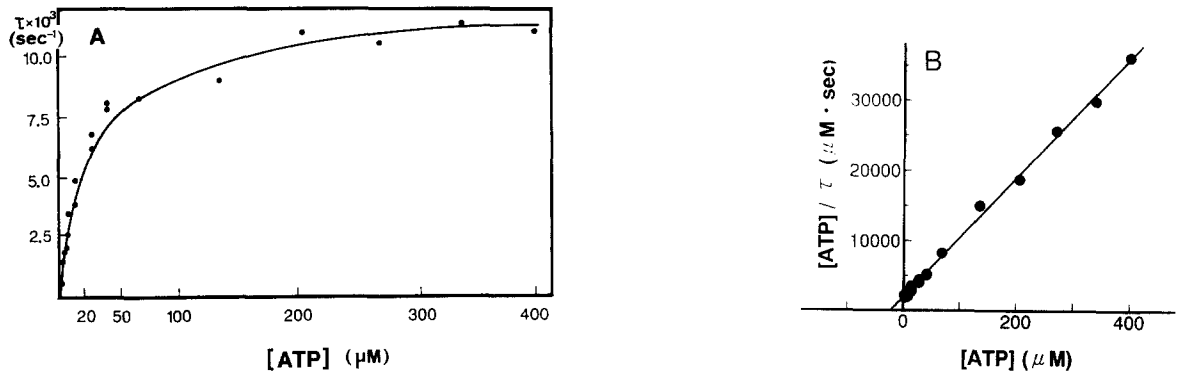

Fig. 2. ATP concentration dependency of the rate constant of inhibition process by NaN_3. (A) Rate constants of NaN_3 inhibition were plotted against ATP concentrations. At ATP concentrations higher than 67.8 μM, the rate constants were derived by fitting the time-course of inhibition as shown in Fig. 1B with an exponential curve. At lower ATP concentrations, the reaction was started by the addition of ATP at the indicated concentrations in the presence of NaN_3. After a period, additional ATP was added to 400 μM and the remaining activity was compared to the activity at 400 μM of ATP without NaN_3. The rate constants were derived from the percentage of inhibition after the period. At ATP concentrations between 1.35 and 40.7 μM, the period was 300 s and between 6.8 to 40.7 μM, the period of 150 s was also examined. (B) A Hanes-Woolf type plotting of (A).

ATPase activity as indicated in the figure legends. 50% inhibition of the ATPase reaction was obtained at 5 μM of NaN_3. In order to examine the effect of ATP or ADP concentration, we employed 500 μM of NaN_3. At a NaN_3 concentration higher than 100 μM, the rate constant of the inhibition process was essentially independent of the NaN_3 concentration.

Centrifugation elution

Centrifugation elution was carried out according to Penefsky [21] using 1 ml tuberculin syringes and Bio-Gel P-10 (Bio-Rad).

Further experimental details are given in the legends.

Results

Relationship between inhibitory effect of NaN_3 and ATP concentration

Fig. 1 shows typical time-courses of inhibitory action of NaN_3 on the ATPase activity of SMP. In Fig. 1A, ATPase reaction was started by addition of ATP (400 μM) and NaN_3 (500 μM) was added subsequently. The addition of NaN_3 caused a slow attenuation of ATP hydrolysis. Final level of attenuation exceeded 95%. This slow attenuation was not an artifact of the response of ATP-regenerating system since oligomycin inhibited the ATPase instantly under the same conditions (data not shown). In Fig. 1B, NaN_3 was added first and ATP was added subsequently. The results clearly indicate that pre-incubation with NaN_3 has no effect for inhibition of ATPase activity in SMP and it inhibits the ATPase only after ATP addition. Furthermore, as shown in Fig. 1C, ATPase activity was attenuated more slowly as ATP concentration was decreased (8 μM).

As described in the Introduction, the ATPase activity of SMP exhibits negative cooperativity and two apparent K_m's of some 10 μM and 200 μM have been calculated [15]. In addition, under single-turnover conditions, a catalytic site with very high affinity has been reported for activated ATPase in SMP [22]. In order to characterize the affinity of the ATP binding which is related to NaN_3 sensitivity and investigate the relationship between the negative cooperativity observed for ATP hydrolysis, we analyzed the time-course of the inhibition process as follows.

A Guggenheim plot of the time-course of the inhibi-

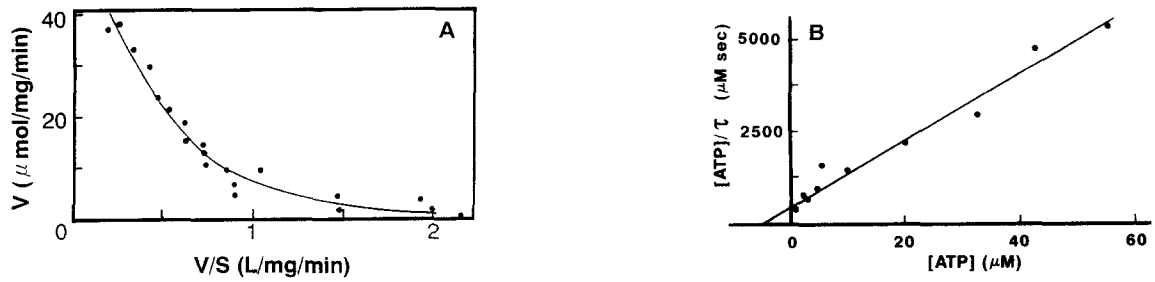

Fig. 3. (A) An Eadie Hofstee plot of ATP hydrolysis by F_1 ATPase. ATP concentration was varied from 2.5 to 2000 μM. (B) ATP concentration dependency of the rate constants of NaN_3 inhibition. NaN_3 was added after ATP and the rate constants were derived by fitting the time-course of inhibition process with an exponential curve at all the ATP concentrations (1.25 to 55 μM) examined.

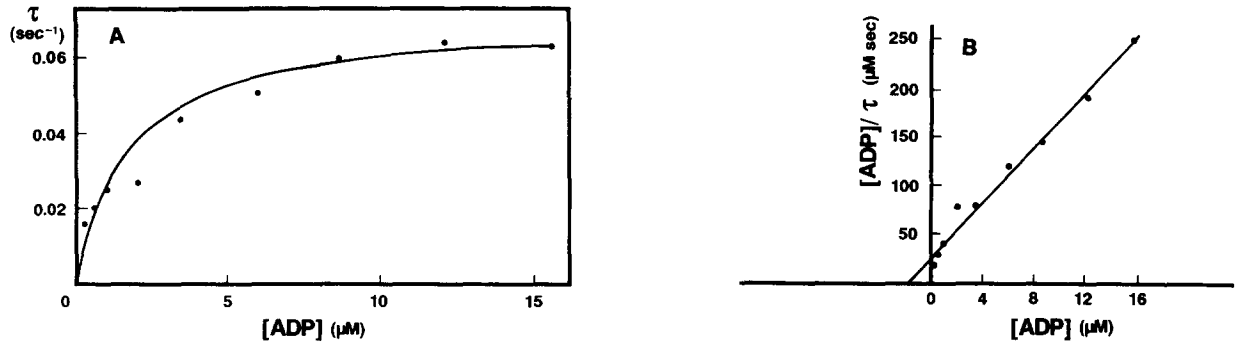

Fig. 4. ADP concentration dependency of the rate constant of inhibition process by NaN_3. (A) Rate constants of the inhibition process were plotted against ADP concentrations. SMP were pre-incubated in 50 mM $TrisSO_4$, 2 mM $MgSO_4$, pH 8.0 with various concentrations of ADP and 500 μM of NaN_3 for 10 to 40 s. Then the remaining ATPase activity was determined by directly adding the aliquots to the ATPase assay mixture containing 400 μM of ATP and 500 μM of NaN_3. The ADP present in the pre-incubation medium was quickly converted to ATP on addition to the ATPase assay mixture and had no effect on the measurement. Rate constants of the inhibition process (τ) were derived by fitting the decrease of ATPase activity with an exponential curve taking 100% of activity with the sample pre-incubated without ADP. (B) A Hanes-Woolf type linear plotting of (A).

tion showed that the process was single exponential. On the assumption that the binding of ATP to the site is in rapid equilibrium compared to the inhibition process and the enzyme which binds ATP to the site is inhibited by NaN_3, the affinity of the site can be estimated from the rate constant of the inhibition process at various ATP concentration. As shown in Fig. 2A, the plot of rate constant (τ) against ATP concentration gave a hyperbolic curve and a Hanes-Woolf type plotting of Fig. 2A (Fig. 2B) gave a straight line. From the plot, the apparent affinity of the binding site which renders NaN_3 sensitivity was estimated to be 14.0 ± 8.7 μM ($\pm$standard deviation) and τ_{max} was 0.012 ± 0.002 s^{-1}. This value was close to the low K_m of some 10 μM previously reported for ATP hydrolysis [15] and far from 10^{-12}M or 200 μM, strongly suggesting that binding of ATP to the site that represents the apparent K_m of some 10 μM is responsible for NaN_3 sensitivity.

The same conclusion was derived as for isolated F_1 ATPase. Our F_1 preparation showed negative cooperativity of ATP hydrolysis characterized by two K_m's 9.0 ± 8.1 μM and 930 ± 210 μM (Fig. 3A). Although it was very slowly inhibited by NaN_3, even in the absence of adenine nucleotides, the inhibition rate was clearly dependent on ATP concentration and half-maximal promotion of NaN_3 sensitivity was attained at 12.6 ± 7.1 μM of ATP (Fig. 3B). This is again well consistent with the low K_m of ATP hydrolysis. However, as the slow inhibition without added adenine nucleotide made some complication in kinetic analyses, we used SMP in the following experiments.

The reversibility of the inhibited state was examined by applying the inhibited SMP to centrifuge columns. It was shown that the presence of only NaN_3 was sufficient to maintain the inhibited state, even if ATP was removed *.

Effect of ADP on NaN_3 sensitivity

The fact that inhibitory action of NaN_3 starts only after ATP addition (Fig. 1B) raised another question whether successive catalytic turnovers are necessary for the inhibition process or not. In order to clarify this point, we pre-incubated SMP with ADP and NaN_3 in 50 mM $TrisSO_4$, 2 mM $MgSO_4$ (pH 8.0) and examined the ATPase activity after centrifuge elution. When SMP were pre-incubated with only ADP (100 μM) for 5 min and passed through a centrifuge column, the ATPase activity was the same as the sample pre-incubated without ADP. When SMP were pre-incubated with ADP and NaN_3 simultaneously, the ATPase activity was inhibited after passage through a centrifuge column containing NaN_3. Like the effect of ATP and NaN_3, the inhibited state was maintained by NaN_3 alone and was reversed when ADP and NaN_3 were removed. From these results, it can be concluded that successive turnovers of ATPase reaction are not necessary for NaN_3 inhibition but the presence of bound ADP is enough.

Then, the affinity of the site for ADP was of interest. Pre-incubation of SMP with NaN_3 and ADP caused time dependent inhibition like that with NaN_3 and

* Using [^{3}H]ATP, we examined the AT(D)P content of the sample after centrifuge elution to remove AT(D)P in the presence of NaN_3. However, although the ATPase activity of the effluent was almost inhibited, we could not detect enough amount of bound label. The use of activated SMP did not improve the data. (Muneyuki, E., and Kamata, H., unpublished results).

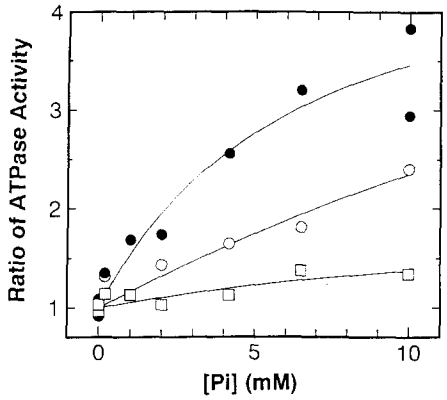

Fig. 5. Effect of inorganic phosphate on NaN_3 sensitivity. SMP were first incubated with indicated concentration of inorganic phosphate for 1 min and then buffer (□), ADP (13 μM) (○) or NaN_3 (500 μM) and ADP (13 μM) (●) were added. Just after 40 s, the ATPase activity was measured by directly adding an aliquot to an ATP-regenerating system containing 500 μM NaN_3 and 400 μM ATP. The actual values corresponding to the 1 in ordinate are 0.673, 0.150 and 0.057 μmol mg^{-1} min^{-1}, for the samples incubated with buffer only (□), ADP (○) and both of ADP and NaN_3 (●), respectively. Ratios of initial velocity with and without inorganic phosphate are plotted.

ATP. Although the data points are somewhat more scattered than the case of ATP, the rate constants (τ) were derived from the time-course of the inhibition on a similar assumption as in the case of ATP binding. In Fig. 4A, the rate constants (τ) is plotted against ADP concentration. A linear plot was made as Fig. 4B and apparent K_d for ADP was calculated to be 1.34 ± 0.45 μM. The corresponding τ_{max} was 0.066 ± 0.008 s^{-1}.

Effect of inorganic phosphate on NaN_3 sensitivity

The results obtained above suggests that F_0F_1 ATPase in SMP becomes sensitive to NaN_3 after bound ATP was hydrolyzed to produce bound ADP on the enzyme. Then, it can be expected that addition of inorganic phosphate affects NaN_3 sensitivity by occupying the γ-phosphate position next to the bound ADP. In order to test this possibility, we examined the effect of inorganic phosphate as shown in Fig. 5. In the absence of inorganic phosphate, pre-incubation with ADP and NaN_3 caused up to 92% inhibition under the experimental conditions here. On the other hand, inorganic phosphate caused more than 200% activation of the residual activity (Fig. 5A, closed circle). Thus, inorganic phosphate prevents NaN_3 inhibition. Similar effects were observed for the inhibitory effect of ADP (Fig. 5A, open circle). These effects can be distinguished from the activating oxianion effect of inorganic phosphate, since inorganic phosphate activates ATPase activity only 30% in the absence of ADP or NaN_3 (Fig. 5A, open squares). Inorganic phosphate may compete with NaN_3 since at higher concentration of NaN_3, the extent of inhibition increased again (data not shown). However, as the relationship between NaN_3 concentration and inhibitory effect was not a simple hyperbolic curve, we did not further analyze it quantitatively.

Discussion

Nucleotide binding site related to NaN_3 sensitivity

F_1 ATPase has six nucleotide binding sites and three of them are exchangeable, while the other three are nonexchangeable [23]. The three exchangeable sites are thought to be catalytic and apparent K_d of 10^{-12} M under single-turnover condition and apparent K_m of 1–30 μM and 100–300 μM are thought to correspond to the binding of substrate ATP to each catalytic site [14]. In this study, it was shown that F_0F_1 ATPase in submitochondrial particles becomes sensitive to NaN_3 after it binds ATP or ADP to some nucleotide binding site whose apparent affinity for ATP was 14.0 ± 8.7 μM. This agreed well with the value previously reported for the lower K_m of ATPase reaction and proton translocation [15]. The fact that ADP can promote the NaN_3 sensitivity as ATP means that successive turnovers of the enzyme are not necessary to become inhibited by NaN_3. The apparent K_d for ADP was 1.34 ± 0.45 μM, which agreed well with the value previously reported by Pedersen [24]. This value may be also compared to the low K_m for ATP synthesis by activated submitochondrial particles [25,26]. These results indicate that the site of ATP binding related to NaN_3 is identical with that of ADP binding and the low K_m mode of ATP hydrolysis corresponds to the low K_m mode of ATP synthesis.

There have been many reports as to the interaction of NaN_3 with F_1 or F_0F_1 ATPase [7,16,17,24,27–32]. Noumi et al. have reported that NaN_3 does not inhibit the uni-site catalysis of EF_1 [17]. Although the meaning of the uni-site catalysis, particularly as for EF_1, is somewhat questionable [33], our present results are consistent with theirs. Recently, Harris has reported the effects of NaN_3 on mitochondrial F_1 ATPase [28]. He also observed that at low ATP concentration, NaN_3 did not inhibit the ATPase activity within 1 min. Although his conclusion is somewhat different from ours, we suspect it might reflect different experimental conditions and direct comparison may be difficult. Our experimental results agree qualitatively well with that obtained by Vasilyeva et al. [16]. They also examined the ATP concentration dependency of the inhibitory effect of NaN_3 but did not analyze the results in relation to negative cooperativity. In the present study, we have demonstrated that the apparent K_d of the ATP binding which promotes NaN_3 inhibition was 14.0 ± 8.7 μM, well consistent with the low K_m observed for negative cooperativity of ATP hydrolysis by F_0F_1 ATPase of SMP. NaN_3 sensitivity was also conferred by binding of ADP and the apparent K_d for ADP agreed with the low K_m for oxidative phospho-

rylation. The bound ADP was quickly released when subjected to centrifuge elution or dilution in the ATPase assay mixture. At least, the nucleotide binding site characterized here is not a non-exchangeable tight binding site and we prefer a view that the site related to NaN_3 inhibition is a catalytic site which participates in steady-state catalysis. However, we also recognize it is possible that there may be a rapidly exchangeable regulatory site and our present conclusion is still tentative. As for the chloroplast enzymes, it was concluded that the ADP binding site related to NaN_3 inhibition was a non-catalytic site [30,34]. As for yeast mitochondrial F_1 ATPase, a mutation in α subunit which was supposed to be at a non-catalytic site was shown to affect NaN_3 sensitivity [35].

Scheme for NaN_3 inhibition

The present results are summarized in a scheme as shown in Fig. 6. In this, ATP first binds to a catalytic site of enzyme with an affinity of 10 μM and is hydrolyzed into ADP and inorganic phosphate (P_i). Under steady-state conditions, ADP dissociates quickly from the enzyme before dissociation of P_i through the upper pathway of the scheme. On the other hand, to a lesser extent, P_i dissociates before ADP and the resultant enzyme · ADP complex, which is less active, is formed through the lower pathway of the scheme. The enzyme · ADP complex should not be totally stable and inactive, but slowly dissociates ADP, since the bound ADP can be easily removed by centrifuge elution. The proportion of the enzyme · ADP complex should be adequately low since the release of P_i before ADP is sufficiently slower than that of ADP before P_i. However, once formed, the enzyme · ADP is sensitive to NaN_3 presumably by forming a ternary enzyme · ADP · NaN_3 complex. The scheme presented here is essentially similar to that proposed by Vasilyeva et al. [16]. The effect of P_i against NaN_3 inhibition may be explained as to shift the equilibrium from enzyme · ADP + P_i to NaN_3 insensitive form of enzyme · ADP · P_i.

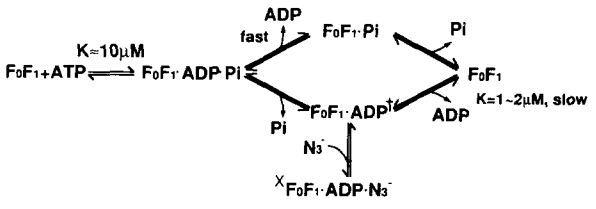

Fig. 6. Scheme for NaN_3 inhibition on the ATPase activity of submitochondrial particles. At the catalytic site which corresponds to the K_m of some 10 μM, the steady-state ATP hydrolysis proceeds mainly through the upper pathway where ADP dissociates quickly before P_i. When P_i dissociates before ADP, the resultant enzyme · ADP complex is less active due to slow release of ADP. NaN_3 acts on this enzyme · ADP complex. See also the text for details.

NaN_3 has been reported as a unidirectional inhibitor: namely, it inhibits only ATP hydrolysis but not ATP synthesis. This may be because of the presence of P_i under ATP synthesis conditions.

Recently, Murataliev et al. have reported that the effect of NaN_3 is related to Mg inhibition [32]. Although Mg ions are not included in the scheme, all the enzyme · adenine nucleotide complex should concomitantly contain Mg ions together. The fact that isolated F_1 is inhibited by NaN_3 even in the absence of ATP or ADP may be explained as that it binds Mg to the site even in the absence of adenine nucleotides. Such enzyme · metal · anion complexes have been proved for creatine kinase by infrared spectroscopy [36]. In the case of SMP, the ATPase activity was insensitive to NaN_3 even in the presence of ADP when Mg was completely removed by EDTA (data not shown), however, the presence of only Mg ions was not sufficient for introduction of NaN_3 sensitivity as described above.

Acknowledgment

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