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Mode of inhibition of sodium azide on H^+-ATPase of *Escherichia coli*

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Sodium azide inhibited multi-site (steady-state) ATPase activity of *E. coli* F_1 more than 90%, but did not affect uni-site (single-site) ATPase activity. Thus azide inhibited multi-site ATPase activity by lowering catalytic cooperativity. Consistent with this observation, azide changed the ligand-induced fluorescence response of aurovertin bound to F_1.

H^+-ATPase; F_1; Uni-site catalysis; Multi-site catalysis; Sodium azide; (*E. Coli*)

1. INTRODUCTION

The H^+-ATPase complex (F_1F_0) of *Escherichia coli* catalyzes ATP synthesis at the terminal step of oxidative phosphorylation (reviews [1-4]). The catalytic portion, F_1, is formed from 5 subunits α, β, γ, δ, and ϵ with an $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometry and acts as an ATPase. The mechanism of ATP hydrolysis by F_1 from mitochondria [5,6] and *E. coli* [7,8] has been studied extensively: the ATP at the first catalytic site is hydrolyzed only slowly ('uni-site' hydrolysis), but on binding of ATP at the second and third sites, the ATP at the first site is hydrolyzed with release of products at maximal velocity ('multi-site' hydrolysis) due to the positive cooperativity between the three catalytic sites. The ratio of the uni- and multi-site rates is 10^6 for mitochondrial F_1 and 10^4-10^5 for *E. coli* F_1.

A useful approach in further studies on the catalytic mechanism of F_1 is the introduction of specific inhibitors. Sodium azide is known to be a potent inhibitor of F_1-ATPase [9,10], although its mode of inhibition has not been studied in detail. Recently we found that F_1 from a mutant strain KF43 (defective in the β-subunit) has a residual multi-site ATPase activity that is insensitive to azide [8]. Senior and co-workers [11,12] showed that the residual multi-site activities of F_1 from *uncA* mutants (defective in the α-subunit) and revertants of *uncA401* were also insensitive to azide. The F_1s from strains KF43 and *uncA401* both had similar uni-site ATPase activity to wild-type F_1, although their multi-site activities were 1-3% of that of wild type. These findings suggest that the interaction between the α- and β-subunits is essential for the positive cooperativity and may be responsible for the azide sensitivity. In this work we examined the effects of azide on the kinetics of uni- and multi-site ATP hydrolyses and on the conformational changes of F_1 on its bindings to ADP, ATP and Mg^{2+}.

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Abbreviations: P_i, inorganic phosphate; $^{32}P_i$, radioactive inorganic phosphate

2. MATERIALS AND METHODS

F_1 was prepared from *E. coli* strain ML308-225 as described [13]. The specific activity of the preparation (multi-site or steady-state activity) was

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75–80 units/mg protein when measured under standard conditions at 37°C, one unit of enzyme being defined as the amount hydrolyzing 1 μmol of ATP/min.

Uni-site hydrolysis (single site or non-steady-state hydrolysis) of ATP was assayed essentially as described by Duncan and Senior [7]: 0.25 μM [γ-^{32}P]ATP was incubated with 0.50 μM F_1 at 25°C in the presence or absence of NaN_3 (200 μM) in 50 mM Tris-SO_4 (pH 8.0) containing 1.0 mM K_2HPO_4 and 0.5 mM $MgSO_4$. Bound [γ-^{32}P]ATP and $^{32}P_i$ or release of [γ-^{32}P]ATP from F_1 were also measured during uni-site hydrolysis. Multi-site rates of ATP hydrolysis were measured at 25°C incubating 0.20 μM F_1 in 40 mM Tris-HCl, pH 8.0, with 4.0 mM [γ-^{32}P]ATP (3×10^{-4} Ci/mmol) and 2.0 mM $MgCl_2$. The radioactivities liberated in 5 and 10 s were determined as described [8]. The fluorescence change of auroventin bound to F_1 was measured at 25°C as follows [14]. Before use, F_1 was passed through a centrifuge column (Sephadex G-50 (fine), 0.5 × 5.5 cm) equilibrated with 10 mM Tris-HCl (pH 7.5), containing 50 mM NaCl and 7.5% glycerol. The reaction was started by addition of F_1 (0.38 μM) to the above buffer containing 1.0 μM aurovertin (total volume, 700 μl) and the fluorescence change was monitored in a Hitachi fluorescence spectrophotometer F-3000 (excitation, 365 nm; emission, 470 nm) on addition of 100 μM ADP, 100 μM ATP and/or 0.5 mM $MgCl_2$ with or without 200 μM NaN_3. Aurovertin D was kindly supplied by Dr M.G. Douglas. Protein concentration was determined as described [15].

3. RESULTS AND DISCUSSION

3.1. *Effect of azide on uni-site hydrolysis*

As shown previously, azide inhibited multi-site ATPase activity (assayed at 37°C) of *E. coli* F_1 non-competitively with a K_i value of 25 μM: 0.5 mM NaN_3 inhibited more than 90% of the activity [9,10]. In this study we found that azide was slightly more inhibitory at 25°C: at this temperature its K_i value was 7 μM and 200 μM NaN_3 caused up to 93% inhibition. On the other hand, azide caused only slight inhibition of uni-site hydrolysis (assayed at 25°C). With or without azide, the enzyme showed similar kinetics of the initial rate of uni-site hydrolysis (measured both as P_i bound to F_1 and as P_i released into the medium), while the rate of the release of P_i from the active site on addition of excess unlabelled ATP (cold-chase experiment) decreased to 60% with the addition of azide.

Table 1

Catalytic properties of F_1 in the presence of NaN_3

Parameter	F_1	F_1 + NaN_3[d]
k_1 ($M^{-1} \cdot s^{-1}$)[a]	3.4×10^4	2.4×10^4
k_{-1} (s^{-1})[a]	$\leqq 6.0 \times 10^{-5}$	$\leqq 6.0 \times 10^{-5}$
$F_1 \cdot ATP/F_1 \cdot ADP \cdot P_i$[b]	2:1	3:2
k_3 (s^{-1})[a]	1.8×10^{-3}	1.4×10^{-3}
k_7 (s^{-1})(multi-site)[c]	78	6.2
k_7/k_3 (promotion of catalysis)	4.3×10^4	4.4×10^3

[a] Values of k_1, k^{-1} and k_3 are rate constants of uni-site catalysis and were calculated as described by Duncan and Senior [7]:

$$F_1 \underset{ATP\ k_{-1}}{\overset{ATP\ k_1}{\rightleftharpoons}} F_1 \cdot ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} F_1 \cdot ADP \cdot P_i \underset{k_{-3}\ P_i}{\overset{k_3\ P_i}{\rightleftharpoons}} F_1 \cdot ADP \underset{ADP}{\overset{ADP}{\rightleftharpoons}} F_1$$

[b] This ratio was estimated from the ratio of F_1 bound ATP ($F_1 \cdot ATP$) to F_1 bound P_i ($F_1 \cdot ADP \cdot P_i$)

[c] Rate of hydrolysis of ATP in multi-site catalysis

[d] 200 μM NaN_3 was used. However, 100 μM NaN_3 gave essentially the same results

Thus F_1 showed a lower value for k_1 (rate of binding of ATP) in the presence of azide, whereas k_{-1} (rate of release of ATP) and k_3 (rate of release of P_i) values were not changed (table 1). Essentially the same results were obtained when 0.10 μM [γ-^{32}P]ATP and 0.20 μM F_1 were used. The amount of ATP bound to F_1 during uni-site hydrolysis decreased slightly with azide, although the amount of bound P_i was the same in the presence or absence of azide. Consequently the ratio of bound ATP ($F_1 \cdot ATP$) to bound P_i ($F_1 \cdot ADP \cdot P_i$) was about 3:2 in the presence of azide compared with 2:1 in the absence of azide, indicating that azide shifted slightly the equilibrium '$F_1 \cdot ATP \rightleftharpoons F_1 \cdot ADP \cdot P_i$' toward $F_1 \cdot ADP \cdot P_i$. The promotion of catalysis was calculated from the rates of release of P_i in uni-site (k_3) and multi-site (k_7) hydrolyses: the value with azide was about one-tenth of that without azide. From these results we conclude that azide inhibited multi-site hydrolysis more than 90%, but did not inhibit uni-site hydrolysis. These results suggest that azide inhibited multi-site activity by lowering the catalytic cooperativity, or transmission of conformational changes between multiple active sites.

3.2. *Effects of azide on the fluorescence of aurovertin bound to F_1*

The effect of azide on transmission of conformational changes was studied with aurovertin bound to the β-subunit as a probe [14,16–18]. Without addition of Mg^{2+}, azide has essentially no effect on the magnitude of the fluorescence of aurovertin bound to F_1 (fig.1A) and F_1 had similar high affinity K_d values (about 0.4 μM, assayed by fluorescence increase) for aurovertin with or without azide, suggesting that azide did not alter the aurovertin binding site. However, as shown in fig.1B, Mg^{2+} slightly quenched the fluorescence of aurovertin bound to F_1 and this quenching was further increased by azide. A similar level of fluorescence quenching was observed on addition of azide followed by Mg^{2+} (fig.1A). These results suggest that the conformation of the β-subunit in the presence of Mg^{2+} was changed on binding of azide. Azide had a similar effect on ADP-induced fluorescence of aurovertin bound to F_1 (fig.1C,D).

The fluorescence of aurovertin bound to F_1 was enhanced by addition of ADP, as shown previously [16]: the fluorescence enhancement showed two

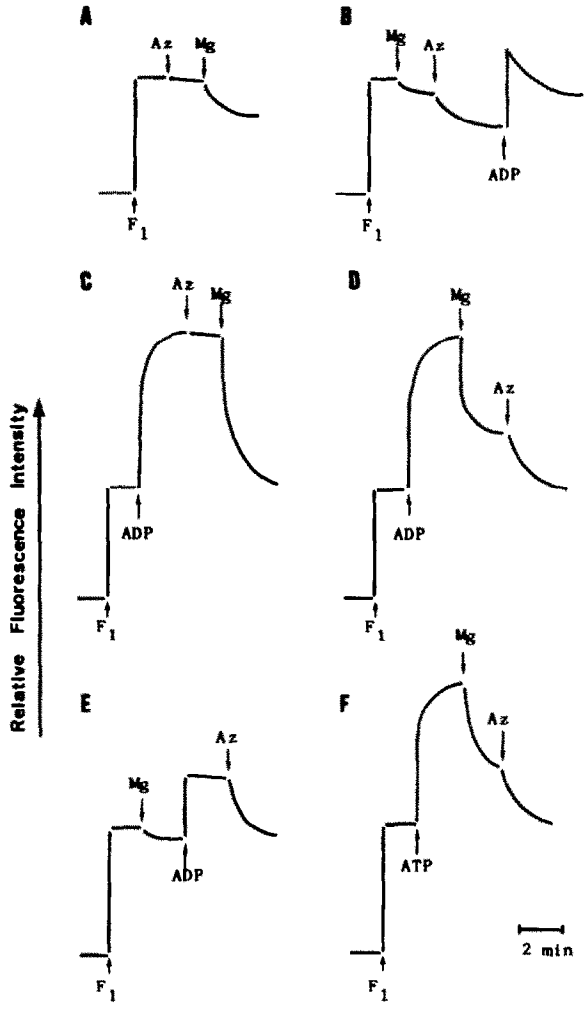

Fig.1. Effects of azide on fluorescence of aurovertin bound to *E. coli* F_1. F_1 (0.38 μM) was added to reaction mixture containing 1.0 μM aurovertin and fluorescence change was monitored. The following compounds were added at the times indicated by arrows: 200 μM NaN_3 (Az), 0.5 mM $MgCl_2$ (Mg), 100 μM ADP or 100 μM ATP (final concentrations). Slight shifts of the base lines upon addition of above compounds were corrected in each experiment. See text for details.

kinetic phases (rapid and slow phases) (fig.1C,D). However, in the presence of Mg^{2+} (added before ADP), only the rapid phase was observed (fig. 1E), suggesting that the two phases represented qualitatively different conformational changes of the β-subunit. The fluorescence enhanced with ADP was quenched gradually in the presence of Mg^{2+} and azide (fig.1B,D,E). As shown in fig.1F,

azide also changed the fluorescence response to ATP. Thus azide altered the nucleotide- as well as the Mg^{2+}-induced conformation of the β-subunit and this alteration was detected by the fluorescence response. Thus azide may affect transmission of conformational change between the ligand site and aurovertin site in the β-subunit. This site-site conformational transmission may be essential for multi-site ATPase activity.

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