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Complete Inhibition and Partial Re-activation of Single F₁-ATPase Molecules by Tentoxin

NEW PROPERTIES OF THE RE-ACTIVATED ENZYME*

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During hydrolysis of ATP, the γ subunit of the rotary motor protein F₁-ATPase rotates within a ring of $\alpha_3\beta_3$ subunits. Tentoxin is a phyto-pathogenic cyclic tetrapeptide, which influences F₁-ATPase activity of sensitive species. At low concentrations, tentoxin inhibits ATP hydrolysis of ensembles of F₁ molecules in solution. At higher concentrations, however, ATP hydrolysis recovers. Here we have examined how tentoxin acts on individual molecules of engineered F₁-ATPase from the thermophilic *Bacillus* PS3 (Groth, G., Hisabori, T., Lill, H., and Bald, D. (2002) *J. Biol. Chem.* 277, 20117–20119). We found that inhibition by tentoxin caused a virtually complete stop of rotation, which was partially relieved at higher tentoxin concentrations. Re-activation, however, was not simply a reversal of inhibition; while the torque appears unaffected as compared with the situation without tentoxin, F₁ under re-activating conditions was less susceptible to inhibitory ADP binding but displayed a large number of short pauses, indicating infringed energy conversion.

F₁-ATPase, the water-soluble part of F₀F₁-ATP synthase, splits ATP into ADP and inorganic phosphate (1, 2). The enzyme is found ubiquitously in bacteria, mitochondria, and chloroplasts and consists of five different subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. Its $\alpha_3\beta_3\gamma$ subcomplex is the minimal assembly capable of continuous hydrolysis of ATP. The α and β subunits, arranged in an alternating manner like the elements of an orange, form a hexagon with the γ subunit in its center (3). Rotation of the γ subunit within the $\alpha_3\beta_3\gamma$ hexagon during

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ATP hydrolysis was first proposed by Boyer and colleagues (4), later supported by biochemical (5) and spectroscopic (6) evidence. Finally, rotation could be observed directly by attaching a fluorescently labeled actin filament as a probe to the γ subunit (7–9).

Tentoxin, a cyclic tetrapeptide produced by phyto-pathogenic fungi of the *Alternaria* species, causes chlorosis in sensitive plant species. It acts as an inhibitor of chloroplast F₁-ATPase (CF₁)¹ from these species but not of the homologous enzymes from chloroplasts of insusceptible plant species or from bacteria or mitochondria (10–12). Inhibition has been attributed to binding of one tentoxin molecule to a high-affinity site with a $K_D < 10^{-8}$ (13). A crystal structure of CF₁ complexed with tentoxin showed that the high-affinity site is located in a cleft at the $\alpha\beta$ subunit interface (14). At higher concentrations of tentoxin, binding of a second and possibly a third molecule to low-affinity sites ($K_D > 10^{-6}$) has been observed (13, 15). Surprisingly, binding of inhibitor molecules to these sites resulted in re-activation of ATP hydrolysis (12, 13, 15). The degree of both inhibition and re-activation of the enzyme varies; whereas spinach CF₁ showed about 80% maximal inhibition and re-activation to about 3-fold its activity in the absence of tentoxin, reduced sensitivity for inhibition and less pronounced re-activation were reported for chimeric complexes constructed from CF₁ and *Rhodospirillum rubrum* F₁-ATPase subunits (16, 17) and for CF₁ treated with synthetic tentoxin analogues (18).

It seems a rather unique property that an alleged inhibitor can exert two such strikingly opposite effects on its target enzyme, and a wealth of effort has been dedicated to the question whether re-activation is a simple reversal of inhibition or other factors are involved. Answering these questions has been hampered by the lack of an experimental system for mutagenesis, overexpression, and assembly of chloroplast F₁. Such methods are well established for an $\alpha_3\beta_3\gamma$ subcomplex of F₁-ATPase from the thermophilic *Bacillus* PS3 (TF₁), however, which additionally is routinely used in single molecule observations of rotation (7, 19). Based on the x-ray structure of tentoxin-bound CF₁, we recently introduced the single point mutation $\beta\text{Ser}^{66} \rightarrow \text{Ala}$ into the thermophilic enzyme. This mutant, referred to as TF₁ βS66A here, displayed similar activity and kinetics as the wild type enzyme. But whereas the wild type enzyme shows very low tentoxin sensitivity at room temperature, sensitivity of the mutant was strongly enhanced. The observed maximal degree of inhibition, 60–70%, was comparable with values reported for CF₁, and recovery of activity was also observed (20).

In the present report, we have used this engineered tentoxin-sensitive F₁-ATPase to examine rotation of single enzyme molecules. For this purpose we adopted the polystyrene-bead detection method of rotation (21), which allows substantially extended observation times as compared with the actin filament technique and has previously successfully been used to examine redox regulation of single F₁-ATPase molecules (22). Our results indicate that tentoxin inhibits by virtually completely blocking rotation. Re-activation restores rotational movement but with altered characteristics as compared with

¹ The abbreviations used are: CF₁, F₁-ATPase from spinach chloroplast; TF₁ βS66A , $\alpha_3\beta_3\gamma$ subcomplex of F₁-ATPase from the thermophilic *Bacillus* PS3 carrying the mutation $\beta\text{Ser}^{66} \rightarrow \text{Ala}$; MOPS, 4-morpholinepropanesulfonic acid.

the situation without tentoxin. The angular speed during any given stretch of continuous rotation was unaffected, indicating that the efficiency of conversion from chemical to mechanical energy for each hydrolyzed ATP molecule was not influenced. However, the occurrence of a large number of short pauses suggests that the probability for this energy conversion process was still reduced in the re-activated state. Furthermore, binding of two or more tentoxin molecules seems to abolish the inhibitory effect of ADP binding to the enzyme.

EXPERIMENTAL PROCEDURES

Protein Preparation—A $\alpha_3\beta_3\gamma$ subcomplex of F_1 -ATPase from the thermophilic *Bacillus* PS3 carrying an affinity tag of 10 histidine residues at the N terminus of the β subunits and the mutation β Ser⁶⁶ → Ala (referred to as TF₁ β S66A) was isolated from *Escherichia coli* strain JM103 Δ uncB-D as described previously (20) with the following modifications: TF₁ β S66A, partly purified with a nickel-nitrilotriacetic acid (Quiagen) affinity column, was loaded onto a Mono Q 5/5 anion exchange column (Amersham Biosciences), equilibrated with 50 mM MOPS/KOH, pH 7.0, containing 50 mM KCl and eluted with a linear gradient from 0 to 250 mM potassium sulfate. Fractions containing TF₁ β S66A were pooled and passed through a Superdex 200 gel filtration column (Amersham Biosciences) equilibrated with 50 mM MOPS/KOH, pH 7.0, containing 50 mM KCl.

Bulk Phase ATP Hydrolysis Activity—ATP hydrolysis was measured with an ATP regenerating system coupled to oxidation of NADH, monitoring the decrease of absorption at 340 nm (23). Samples were preincubated for 2 h with tentoxin at the concentrations indicated in Fig. 1, and ATP hydrolysis was recorded in the presence of the same concentrations of tentoxin.

Rotation Assay—TF₁ β S66A was biotinylated at a single cysteine residue in the γ subunit using biotin-(PEAC)₅-maleimide (Dojindo) as described previously (7, 22). The biotinylated enzyme was preincubated for 2 h in 10 mM MOPS/KOH, pH 7.0, 50 mM KCl (Buffer A) containing 50 μ M or 1 mM tentoxin (diluted from 100-fold concentrated stock in ethanol). All buffers for the subsequent steps of sample preparation and microscopic observation contained the required concentration tentoxin or 1% ethanol as control. 15 μ l of this mixture (20–50 nM TF₁ β S66A) was infused into a flow cell, constructed from microscope cover slips as described (7), and incubated for 2 min to allow for immobilization of the enzyme on the glass surface. The flow cell was washed with 100 μ l of Buffer A containing 10 mg/ml bovine serum albumin (Buffer B), a suspension of streptavidin-coated polystyrene beads (Bangs Laboratories, diameter 500 nm, ~200 pM) in Buffer B was infused and incubated for 20 min. Subsequently, 100 μ l of reaction buffer (Buffer B supplemented with 2 mM ATP, 4 mM MgCl₂, 2.5 mM phosphoenolpyruvate, and 0.1 mg/ml pyruvate kinase (Roche Applied Science)) was infused and microscopic observation was started. Rotation of beads was observed with an IX70 microscope (Olympus). Images were recorded with a charge-coupled device (Dage-MTI) and stored in a computer. Image analysis was done using the CREST IMAGE software provided by R. Yasuda (24). For each data set 100 molecules were recorded for a 5-min period and analyzed; in all cases two beads were bound to one F_1 -ATPase molecule. 20–30 additional molecules per data set, either detached from the slide glass during the observation period, were clearly obstructed by contact of the beads with the glass surface or were bound to more than two beads. Still, inclusion of these molecules into the analysis did not significantly change the results. For a detailed description of the method, refer to Ref. 21.

To monitor the effect of different tentoxin concentrations on the same F_1 -ATPase molecule, we screened a flow cell containing the reaction buffer described above without tentoxin for 60 min and observed each rotating molecule for 5 min. Then buffer containing 50 μ M tentoxin was infused, and the same molecules were recorded again for 5 min, 90–150 min after tentoxin addition. Subsequently, this procedure was repeated using reaction buffer with 1 mM tentoxin. To prevent the sample from drying out, fresh buffer was infused in 30-min intervals without changing the tentoxin concentration.

RESULTS AND DISCUSSION

Time Course of Rotation in the Absence and Presence of Tentoxin—The tentoxin-sensitive thermophilic F_1 -ATPase TF₁ β S66A showed maximal inhibition of bulk phase ATP hydrolysis activity in the presence of 50 μ M tentoxin. Higher inhibitor concentrations, up to the solubility maximum of 1 mM tentoxin,

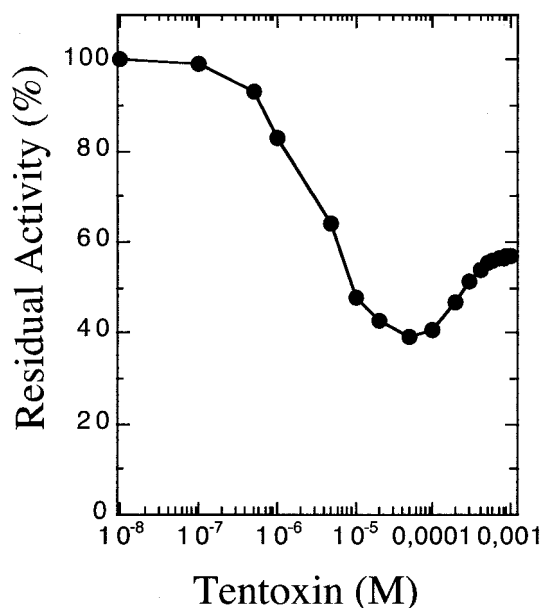


FIG. 1. Bulk phase ATP hydrolysis activity of tentoxin-sensitive thermophilic F_1 -ATPase (TF₁ β S66A) depending on the tentoxin concentration (see also Ref. 20). The specific activity in the absence of tentoxin, 10 units/mg, was taken as 100% value.

re-activated the enzyme (Fig. 1; see also Ref. 20). In this study we examine tentoxin inhibition and reactivation of F_1 -ATPase on the single molecule level. In Fig. 2, time courses of the rotation of TF₁ β S66A are shown that have been recorded employing the polystyrene bead detection method (21). As already found with bulk phase ATP hydrolysis measurements (20), introduction of the mutation β S66A *per se* did also not influence the rotational movement, since no significant differences were observed between TF₁ β S66A and the wild-type enzyme. Rotation in the absence of tentoxin was not continuous but interrupted by pauses of varying length in the tens of seconds time scale (Fig. 2A; Ref. 25). In the presence of 1 mM tentoxin this pattern changed significantly. As depicted in Fig. 2C, the inhibitor caused a decline of the number of long pauses and substantially decreased the average slope of the traces. In the presence of 50 μ M tentoxin we found a mixture of molecules with the rotation/pause pattern characteristic for the absence of tentoxin and molecules showing a strongly reduced average rotational rate (Fig. 2B).

To ensure compatibility of single molecule observations with measurements carried out on ensembles of enzymes, we compared bulk phase ATP hydrolysis activity with the averaged rotational speed derived from single molecule assays. We calculated the average number of revolutions within the 5-min observation period for 100 particles: 225 ± 35 without tentoxin and 115 ± 15 at 1 mM tentoxin. This ratio of 100:51 is comparable with the corresponding values for hydrolysis activity in bulk phase assay, which was determined as 100:55.

Extension of Observation Time Reveals Complete Stop of Rotation of F_1 —Earlier experiments on more sensitive F_1 species indicated that tentoxin inhibition may approach a complete cease of ATP hydrolysis (18), which should be accompanied by a complete halt of rotation. With the thermophilic mutant employed here, the onset of reactivation obscured this effect in bulk phase measurements. We therefore attempted to resolve the inhibitory and re-activating effects by buffer exchange experiments in the single molecule assay. Applying a strongly extended time frame, the same particle was first observed in the absence of tentoxin (phase 1) and subsequently in the presence of 50 μ M (phase 2) and 1 mM tentoxin (phase 3).

FIG. 2. Rotation of single F_1 -ATPase molecules in the absence and presence of tentoxin. Typical time courses of the rotation observed for particles in the absence of tentoxin (A), compared with different particles in the presence of 50 μM (B) and 1 mM tentoxin (C), are shown. A total of 100 particles, with two beads attached each, were recorded under each condition for a 5-min period.

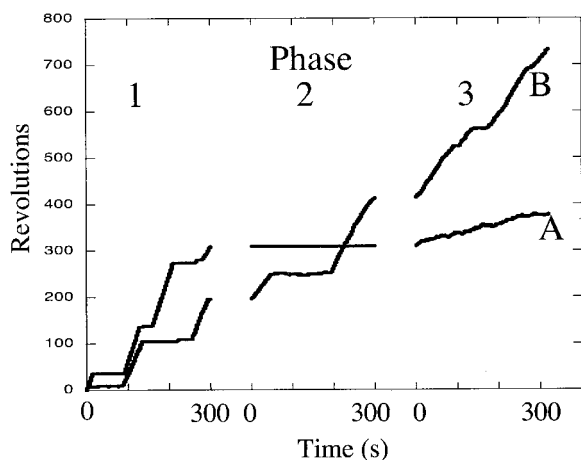
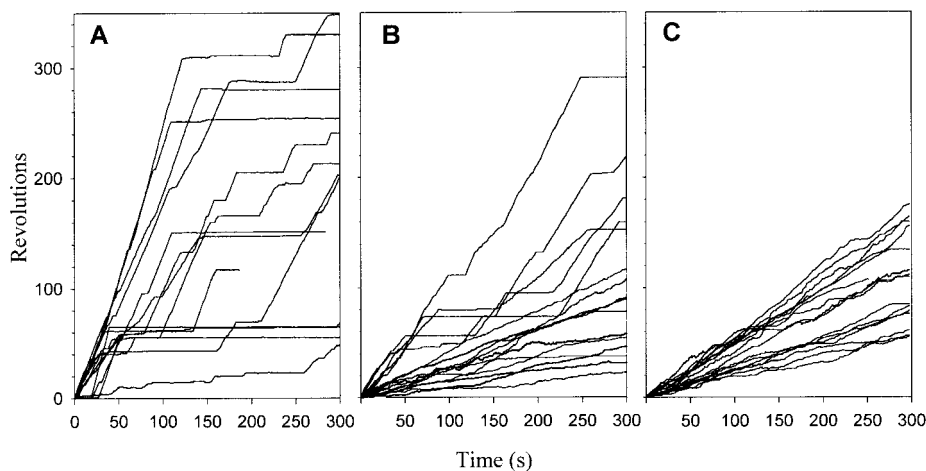


FIG. 3. Effect of tentoxin infusion on rotation. Individual F_1 -ATPase molecules were recorded for a 5-min period in the absence of tentoxin (phase 1). After infusion of 50 μM tentoxin and an incubation period of ~ 2 h, the same particles were observed again (phase 2). Finally tentoxin addition and incubation were repeated using 1 mM tentoxin (phase 3). Trace A represents a typical result (total 14). As a control, buffer without tentoxin was infused (trace B, total 11). For details refer to "Experimental Procedures."

The majority of observed complexes came to a full stop in phase 2, lasting the whole observation period of 5 min (Fig. 3). This demonstrates that inhibition of F_1 -ATPase by tentoxin implies the anticipated complete arrest of the rotational movement. Apparently, the inhibitor molecule does not detach and rapidly re-attach to the enzyme molecule as this should lead to alternating periods of rotation and pauses. Consistent with this observation, a very low k_{off} rate for tentoxin has been indicated by bulk phase experiments; after preincubation with 50 μM tentoxin and subsequent 50-fold dilution of aliquots in buffer either without tentoxin or containing 50 μM tentoxin, we could not detect significant differences in hydrolysis activity between the two samples for 20 min (data not shown, see also Ref. 13). In the single molecule experiments shown in Fig. 3, upon further increase of the tentoxin concentration (phase 3), rotation resumed, but now in the mode already observed in Fig. 2C, *i.e.* at slower average speed as compared with the situation without any tentoxin present.

Thus, we identified three different modes of rotational operation: uninhibited F_1 showed the highest averaged rotational speed, interrupted by a few, relative long pausing periods (phase 1, Fig. 2A, and fraction of traces in Fig. 2B). At maximal inhibition, the majority of particles completely arrests rotation (phase 2). Under conditions of reactivation, rotation resumes at

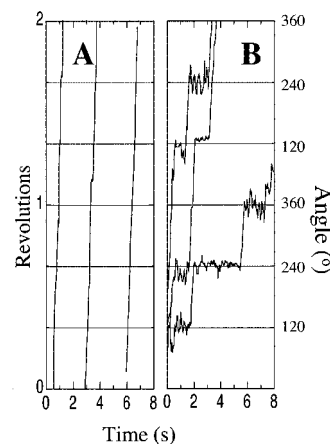


FIG. 4. Angular position of pauses. Time course traces of the rotary movement (as shown in Fig. 2) were enlarged to analyze 1–2 revolutions displayed by individual TF₁ β S66A molecules within several seconds in the absence of tentoxin (A) or in the presence of 1 mM tentoxin (B).

lower average speed and with much lesser pausing periods (phase 3, Fig. 2C, and fraction of traces in Fig. 2B).

In buffer exchange experiments, the large number of enzymes that either detached from or made nonspecific contact to the surface severely complicated quantitative examination. For these reasons we focused on data as depicted in Fig. 2 for further analysis.

Re-activation Partially Restores Rotation—To address the question of how a virtually complete stop of rotation can be overruled by binding of additional inhibitor molecules to F_1 -ATPase, we examined a small number of revolutions displayed by single enzyme molecules at higher time resolution (Fig. 4). As in earlier experiments, in the absence of the inhibitor quasi-continuous rotation is observed (Refs. 7, 22, and 25; Fig. 4A). In the presence of 1 mM tentoxin, the higher time resolution unraveled a strikingly different picture; short periods of rotation were interrupted by well defined stops (Fig. 4B). During rotation, there was no difference in angular speed detectable in the presence or absence of tentoxin. During stops, only Brownian motion was detectable, and resting positions were clearly located at discrete 120, 240, or 360° angular positions. The smaller apparent slope observed at lower time resolution (Fig. 2, B and C) can thus be explained by the increasing number of short pauses. Although any full revolution of F_1 always involves stops of the γ subunit at the three β subunits, separated by 120° angles, at saturating ATP concentrations these three distinct stops are not resolved by this observation technique.

Stop positions separated by 120° steps have been resolved, however, under conditions of ATP shortage and have thus been characterized as ATP waiting positions (24, 26). The speed of any particular 120° rotational step of the reactivated enzyme, a measure for the torque of the movement, was not significantly different from that of the quasi-continuous rotation of the uninhibited enzyme (*cf.* Fig. 4). It can thus be concluded that in the reactivated state, conversion of chemical energy provided by an ATP molecule into the mechanical energy of rotation remains as efficient as in the absence of tentoxin. Instead, the large number of short periods of inactivity suggests that the bound inhibitor molecules interfere with the process of energy delivery necessary to drive rotation, which implies binding of ATP to an empty nucleotide binding site and subsequent conformational change. We regard it as unlikely that switching from stop to rotation is due to rapid detachment and re-attachment of tentoxin to its low-affinity binding site, as we could measure reactivation in bulk phase experiments for at least 10 min after preincubation with tentoxin but without tentoxin in the ATP hydrolysis reaction mixture (data not shown). This finding also rules out that effects observed at higher tentoxin concentrations are due to competition of tentoxin molecules for just one binding site.

Tentoxin Redistributes Two Different Pause Populations—Comparable to reported values (25), in the absence of tentoxin the enzyme rested about 1.5% of its time in short pauses (1–4 s), tentatively attributed to the ATP waiting state (see above). This number increased to 18% in the presence of 1 mM tentoxin. As mentioned before and shown in Fig. 2, we observed an opposite effect on the long pauses (>20 s); here the resting time decreased from 40% (without tentoxin) to 16% (1 mM tentoxin).

These long pauses are caused by the so-called ADP inhibited state of the enzyme (25). This state is caused by ADP, formed by hydrolysis of ATP, which cannot detach from the enzyme, but becomes entrapped in a nucleotide-binding site (27–32). The decline of pauses corresponding to the ADP inhibited state in the presence of tentoxin strongly suggests that tentoxin is relieving ADP inhibition. Similar results have previously been reported for F_1 -ATPase in the presence lauryl-dodecyl aminoxide (25), which also activates by relieving ADP inhibition (29). Accordingly, we found that the stimulatory effect of 0.4% lauryl-dodecyl aminoxide or 100 mM potassium sulfate, another reagent known to relieve ADP inhibition (33, 34), was strongly reduced in the presence of >50 μ M tentoxin (data not shown).

Tentoxin thus redistributes two different pause populations, and in this respect, re-activation is not a simple reversal of inhibition. Experiments to clarify whether the long and short pauses correspond to the same reaction intermediate or if different steps are involved are under way in our laboratory.

Feasibility of Single-molecule Detection Methods for Investigation of Enzyme Regulation—Our results, demonstrating that rotation ceases upon inhibition by tentoxin and resumes with new characteristics upon re-activation, underscore the importance of investigating the interactions of single enzyme molecules. Previously, we examined the redox regulation of engi-

neered F_1 -ATPase and found under oxidized, inhibited conditions a high number of very long (>2 min) pauses, interrupted by periods of normal rotation, attributed to two states of the oxidized enzyme (22). Here, observations of enzyme/inhibitor interactions on the single-molecule level show that activity of an enzyme molecule can be completely blocked and subsequently recover, but with new characteristics, shedding light on so far poorly understood observations obtained from ensembles.

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