*Biochimica et Biophysica Acta*, 504 (1978) 136—141


BBA 47562

# THE STIMULATION OF COUPLING FACTOR 1 ATPase BY TENTOXIN

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(Received January 23rd, 1978)

## Summary

Tentoxin at 10—1000 μM causes a marked species-selective stimulation of coupling factor 1 $Ca^{2+}$-dependent ATPase activity ($K_a$ $6.3 \cdot 10^3$ $M^{-1}$). This effect decreases the $K_m$ for ATP to about 0.3 mM and increases $V$ 2.75-fold. Above 1.6 μM tentoxin the rate of coupled electron transport was reduced to basal without uncoupling.

## Introduction

In previous work the species-selective inhibition of coupling factor 1 ($CF_1$) ATPase and photophosphorylation by tentoxin (cyclo(-L-leucyl-*N*-methyl-(*Z*)-dehydrophenylalanylglycyl-*N*-methyl-L-alanyl-); molecular weight 414) at concentrations of 2—100 nM was characterized [1]. This inhibition is uncompetitive [2] and involves binding to a single site on the α and/or β subunits with an apparent constant ($K_a$) of $2 \cdot 10^8$ $M^{-1}$. In extending these studies to concentrations in the 10—1000 μM range, we have observed a second species-selective effect in which ATPase activity, but not photophosphorylation, reappears with a consequent decrease in the $K_m$ for ATP and an increase in $V$. This paper presents results characterizing this stimulatory effect of tentoxin.

## Materials and Methods

ATP, DCC-treated trypsin and soybean trypsin inhibitor were supplied by Sigma Chemical Company *. Electrophoretic materials were purchased from Bio-Rad.

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Abbreviations: $CF_1$, coupling factor 1; DCC, *N,N*-dicyclohexacarbodiimide.

* Mention of companies or commercial products does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

Tentoxin was prepared from culture filtrates of *Alternaria alternata* as described [3]. In some experiments, synthetic tentoxin was used to obviate the possibility that the effects were due to dihydrotentoxin, a contaminant which occurs in some lots of biosynthetic material.

Preparations of chloroplast coupling factor 1 sensitive to tentoxin were prepared from *Lactuca sativa* var. *longifolia* (romaine lettuce), *Spinacia oleracea* (spinach) and *Nicotiana solanifolia* using methods adapted from those of Lien and Racker [4]. *Raphanus sativus* cv. comet (radish) and *Nicotiana paniculata* were used as sources of $CF_1$ that are insensitive to 2—100 nM tentoxin. When necessary, additional purification of $CF_1$ was achieved by electrophoresis using 4% polyacrylamide gels. The ATPase bands in these gels were stained with calcium phosphate according to the protocol of Horak and Hill [5], cut out with a razor blade and ground in 40 mM Tricine/NaOH buffer, pH 8.0. The enzyme was recovered in the supernatant fraction following centrifugation.

Unless otherwise stated, $CF_1$ was activated by gentle trypsin digestion following the method of Lien and Racker [4]. In some experiments a more drastic trypsin treatment was employed which removed the $\gamma$, $\delta$ and $\epsilon$ subunits [6]. Final purification was achieved by electrophoresis.

Protein was determined using the method of Lowry et al. [7]. ATPase activities and rates of coupled electron transport were determined by measuring $PO_4^{3-}$ release [2] and light-induced oxygen uptake [1], respectively.

## Results

The response of lettuce $CF_1$ ATPase to tentoxin concentrations of 1—2 mM in the presence of 4 mM ATP is indicated in Fig. 1. It is important to note that the tentoxin concentration scale is logarithmic and that later results will demonstrate that enzymatic activity is jointly dependent upon tentoxin and

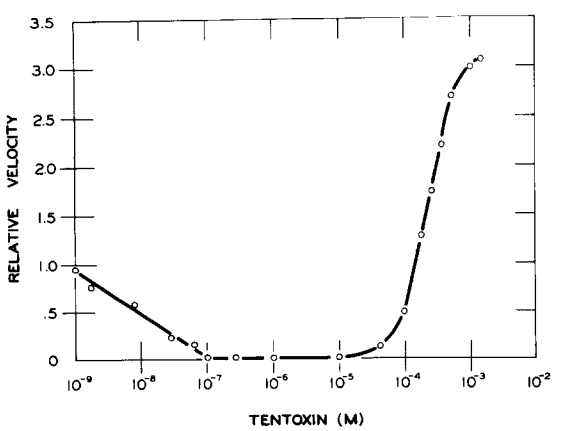


**Fig. 1. Relative velocity of lettuce $CF_1$ ATPase (activated by mild trypsin digestion) vs. tentoxin concentration. Substrates employed were 4 mM ATP and 10 mM $Ca^{2+}$. The reaction mixture was incubated at 37°C using 10 μg enzyme (spec. act. 17.3 μM $PO_4^{3-} \cdot min^{-1} \cdot mg^{-1}$). Relative velocity is defined as the observed rate/rate in the absence of tentoxin.**

ATP concentrations. The $CF_1$ ATPases of lettuce, spinach and *N. solanifolia* were stimulated by tentoxin at concentrations $\geqslant 0.2$ mM and inhibited by concentrations of 10—100 nM. Neither effect was observed when radish or *N. paniculata* $CF_1$ were employed. Similar results were obtained with synthetic tentoxin. When lettuce $CF_1$ was incubated with 240 $\mu$M tentoxin, 2 mM ADP and 10 mM $Ca^{2+}$ no release of phosphate was detected, thus ruling out ADP hydrolysis as a contributor to the observed stimulation.

To determine if photophosphorylation was similarly stimulated, coupled electron transport rates of lettuce chloroplasts were measured in the presence of tentoxin as high as 240 $\mu$M. Above 1.6 $\mu$M tentoxin, the rate of electron transport was reduced to the basal rate without uncoupling. When the $Mg^{2+}$-dependent ATPase of lettuce and *N. solanifolia* chloroplast membranes was measured (25 $\mu$g chlorophyll/assay, 2 mM $Mg^{2+}$, 0.5 mM ATP), a 2.3-fold stimulation occurred at 80 $\mu$M tentoxin. Comparable results were obtained with spinach; however chloroplast membranes from radish did not exhibit this effect.

The response of lettuce $CF_1$ ATPase to different ATP and tentoxin concentrations is illustrated by a double reciprocal plot (Fig. 2). At 60 $\mu$M tentoxin the plot is nonlinear with the observed inhibition increasing sharply at >2 mM ATP. A less marked non-linearity was also observed at 120 $\mu$M tentoxin. Similar non-linearities were observed in an additional experiment.

Fig. 3A and B presents similar experiments using the enzyme stripped of its $\gamma$, $\delta$ and $\epsilon$ subunits. Restoration of activity appears to be due to a decrease in $K_m$ from approx. 1 to 0.3 mM and a 2.75-fold increase in $V$. The $1/V$ intercept plotted against tentoxin concentration formed a hyperbola with half maximal stimulation at 160 $\mu$M tentoxin (apparent $K_a$ of $6.3 \cdot 10^3$ $M^{-1}$).

The non-linearities encountered in double reciprocal plots when $CF_1$ was incompletely trypsinized led us to a preliminary examination of the response of $CF_1$ ATPase to tentoxin and exogenously added $\epsilon$ subunit. The addition of 20 $\mu$g of 7 M urea-denaturated $CF_1$ as a source of $\epsilon$ subunit [8] resulted in 90%

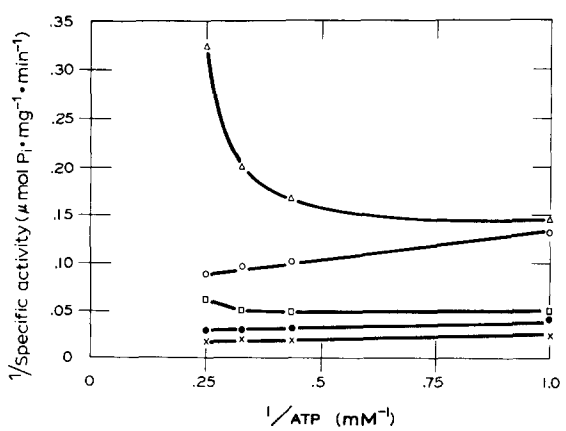


Fig. 2. Double reciprocal plot of $CF_1$ ATPase (activated by mild trypsin digestion) vs. ATP concentration. The treatment lacking tentoxin is represented by (○), 60 $\mu$M tentoxin (△), 120 $\mu$M tentoxin (□), 190 $\mu$M tentoxin (●), and 360 $\mu$M tentoxin (X).

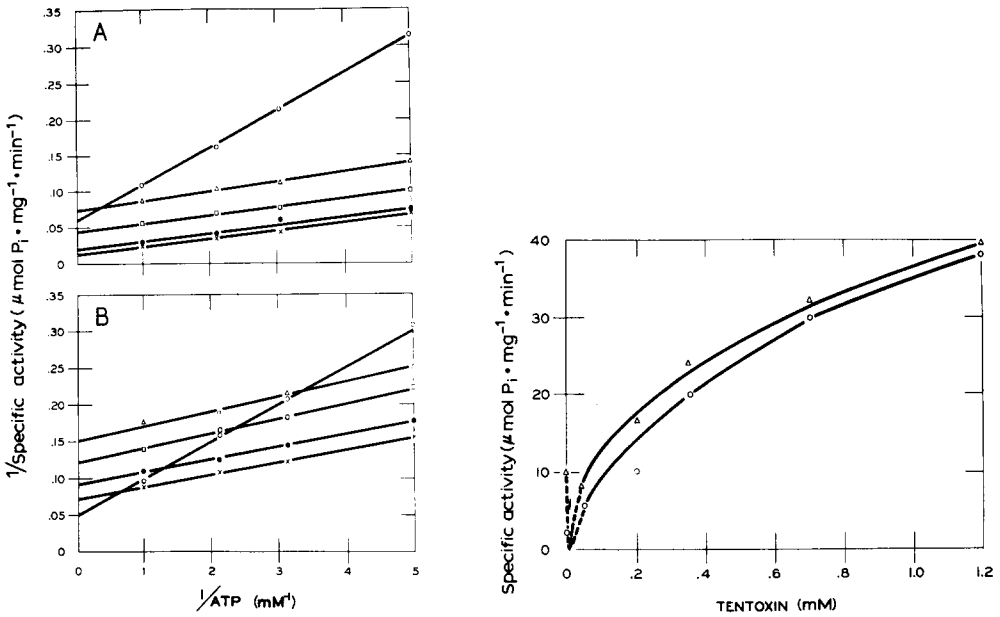


Fig. 3. (A) Double reciprocal plot of specific activity of $CF_1$ (trypsin treated to remove the $\gamma$, $\delta$ and $\epsilon$ subunits and purified by electrophoresis) vs. ATP concentration at various tentoxin concentrations. The incubation mixture contained 3 $\mu$g protein and 10 mM $Ca^{2+}$. The reaction was allowed to proceed for 20 min at 37°C. The treatment lacking tentoxin is represented by (○), 60 $\mu$M tentoxin (△), 120 $\mu$M tentoxin (□), 240 $\mu$M tentoxin (●), and 480 $\mu$M tentoxin (X). The apparent $K_m$ values for ATP are 1 mM in the absence of tentoxin and 0.3 mM at 480 $\mu$M tentoxin. Half maximal stimulation is observed at 160 $\mu$M tentoxin. (B) The enzyme and reaction conditions are as described for (A). The treatment lacking tentoxin is represented by (○), 3 $\mu$M tentoxin (△), 6 $\mu$M tentoxin (□), 12 $\mu$M tentoxin (●), and 24 $\mu$M tentoxin (X). The apparent $K_m$ in the absence of tentoxin is 1 mM.

Fig. 4. Effect of tentoxin on $CF_1$ ATPase. Enzyme activities prior to (○) and following (△) mild trypsin digestion are presented. The initial inhibition shown on the graph is based on data from many previous experiments (refs. 1, 2, 9 and Fig. 1) in which complete inhibition is encountered at 100 nM tentoxin.

inhibition of $Ca^{2+}$-dependent ATPase activity. No such inhibition was observed when $CF_1$ lacking $\gamma$, $\delta$ and $\epsilon$ subunits was employed as the enzyme source. Adding 120 or 480 $\mu$M tentoxin to controls and $\epsilon$ subunit-treated assays reduced the observed inhibitions to 66 and 36%, respectively. The ability of the $\epsilon$ subunit to inhibit tentoxin-stimulated ATPase was determined by adding 3—50 $\mu$g of urea-denaturated $CF_1$ to $CF_1$ in the presence of 180 $\mu$M tentoxin. The inhibition in this case increased smoothly from 5 to 72%.

Fig. 4 presents the effects of tentoxin on $CF_1$ ATPase and the same enzyme preparation prior to activation with trypsin (the solubility limit of tentoxin ($\approx$2.4 mM) prevented extension of the curve to higher concentrations). The unactivated enzyme reached 97% of the activity achieved with the activated enzyme indicating that tentoxin can overcome the latency of the $Ca^{2+}$-dependent ATPase. The initial sharp inhibition (Fig. 4) has been inserted in the absence of specific experimental data because it has been amply demonstrated that $Ca^{2+}$-dependent ATPase is completely inhibited at tentoxin concentrations not distinguishable from 0 on the scale used. (See also refs. 1, 2, 9 and Fig. 1.)

Stimulation of ATPase by tentoxin was shown to be reversible in the follow-

ing manner: Lettuce $CF_1$ (50 μg in 4 ml Tricine/NaOH buffer, pH 8.0) was incubated with 100 μM tentoxin for 2 h, then assayed for $Ca^{2+}$-dependent ATPase activity. The preparation was then subjected to diafiltration using 10 volumes of buffer, and reassayed without additional tentoxin, or with 100 μM tentoxin. The enzyme preparation lacking exogenous tentoxin had 6% of the activity of the two preparations at 100 μM tentoxin.

## Discussion

The marked stimulation of $Ca^{2+}$-dependent ATPase activity observed at 10—1000 μM tentoxin arises from the appearance of an enzyme form whose $K_a$ for ATP and $V$ are increased. This effect of tentoxin exhibits the properties of a saturable binding site with an apparent affinity constant of $6.3 \cdot 10^3$ $M^{-1}$, which contrasts sharply with the $K_a$ of $2 \cdot 10^8$ $M^{-1}$ observed for the inhibitory site. These two effects are linked to a considerable degree since 10—1000 μM tentoxin does not stimulate ATPase of species insensitive to inhibition at 2—100 nM (radish and *N. paniculata*). The sites responsible for both effects must reside on the α and/or β subunits since the complex of these subunits obtained after extensive trypsin digestion exhibits both effects. These correlations and the observation that the tentoxin binding site ($K_a = 2 \cdot 10^8$ $M^{-1}$) responsible for inhibition might be shared between the α and β subunits [9] suggest that the stimulatory effect ($K_a \approx 6.3 \cdot 10^3$ $M^{-1}$) could arise through binding of a tentoxin molecule to each of two subsites. This possibility is supported by energy considerations, i.e., the inhibitory $K_a$ is equivalent to −11.8 kcal. while the apparent stimulatory $K_a$ is about −5.4 kcal.

Since 10—1000 μM tentoxin stimulated $Mg^{2+}$ + $Ca^{2+}$-dependent ATPase of chloroplast membranes and failed to restore coupled electron transport rates in chloroplasts, it appears that tentoxin might circumvent the mechanisms which prevent ATP hydrolysis. This hypothesis is also supported by the observation that both 'native' and trypsin-treated enzyme preparations responded to 10—1000 μM tentoxin similarly (Fig. 4). Tentoxin might thus prove to be a useful tool in investigating the mechanism responsible for latency of $CF_1$ ATPase.

**Note added in proof** (Received July 10th, 1978)

The tentoxin analogs, Sar'-tentoxin and D-MeAla'tentoxin, at high concentrations will also stimulate $CF_1$ ATPase (Steele, J.A., Uchytil, T.F. and Durbin, R.D. (1978) Biochem. Biophys. Res. Commun., in the press).

## Acknowledgements

Research cooperative with the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and the Agricultural Research Service, U.S. Department of Agriculture. The research was supported, in part, by a grant from the National Institute of General Medical Sciences (GM 19311).

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