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THE BINDING OF TENTOXIN TO A TRYPTIC DIGEST OF CHLOROPLAST COUPLING FACTOR 1

JOHN A. STEELE, T. F. UCHYTIL and R. D. DURBIN

Plant Disease Resistance Research Unit, ARS, USDA, Department of Plant Pathology, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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SUMMARY

The binding of tentoxin to lettuce chloroplast coupling factor 1 and its inhibition of Ca^{+2}-dependent ATPase involves the α and β subunits which remain after trypsin treatment. The tentoxin-binding properties of the digest are not greatly altered from those previously reported for the untreated protein.

INTRODUCTION

Tentoxin [cyclo-L-leucyl-*N*-methyl-(Z)-dehydrophenylalanyl-glycyl-*N*-methyl-L-alanyl)], a phytotoxin produced by the fungus *Alternaria tenuis* [1, 2], is a species specific inhibitor of CF_1*. In lettuce, it inhibits photophosphorylation and the Ca^{+2}-dependent ATPase of CF_1 by binding to a single site with an affinity constant of about $2 \cdot 10^8\ M^{-1}$ [3]. In radish photophosphorylation and CF_1 ATPase are, respectively, 20- and 200-fold less sensitive to tentoxin than in lettuce, and solubilized radish CF_1 binds tentoxin only weakly (affinity constant $< 10^4\ M^{-1}$).

Solublized spinach and bean CF_1 contain five different polypeptides designated as α, β, γ, δ, and ε with approximate molecular weights of 59 000, 56 000, 37 000, 17 500, and 13 000, respectively [4, 5]. Following prolonged trypsin digestion, spinach CF_1 yields a Ca^{+2}-dependent ATPase consisting of a somewhat modified complex of α and β subunits [6]. In order to localize the tentoxin site and to determine if it emulates the ε subunit we have examined the interaction between a similar trypsin digest of lettuce CF_1 and tentoxin.

MATERIALS AND METHODS

Measurements of Ca^{+2}-dependent ATPase and tentoxin binding were performed as previously described [3, 7]. Protein was determined by the method of Lowry et al. [8]. Tentoxin, tritiated tentoxin, and lettuce CF_1 were prepared as previously described [3, 9].

* Chloroplast coupling factor 1, CF_1.

Lettuce CF_1 (12 mg) was subjected to trypsin digestion following the method of Deters et al. [6]. The specific activity and sensitivity to tentoxin (200 ng/ml) of the Ca^{+2}-dependent ATPase were determined [3] at 0, 0.5, 1.5, 2.5, 3.5, 4.5 and 6 h of trypsin treatment. After 6 h of digestion, an equal volume of saturated ammonium sulfate was added to the reaction mixture and the protein recovered by centrifugation. The pellet was dissolved in 1 ml of 10 mM Tricine, pH 8.0, 2 mM EDTA, 1 mM ATP and 100 mM NaCl and chromatographed at room temperature on a column of Sephadex G-200* (1 ×45 cm) equilibrated with the same buffer. Each fraction (1 ml) was assayed for ATPase activity and the active fractions (numbers 16–22) were pooled. The protein (5.7 mg) was precipitated by the addition of an equal volume of saturated ammonium sulfate and the suspension stored at 4 °C. Protein was collected by centrifugation, brought to room temperature, dissolved in 10 mM Tricine buffer, pH 8.0, and desalted on a Biogel P-2 column (1 ×20 cm). When required, protein solutions were concentrated using a Minicon B-15 concentrator (Amicon Corp.).

Following purification of the digested CF_1, inhibition curves for the Ca^{+2}-dependent ATPase were obtained for tentoxin concentrations from 0 to 0.24 μM and analyzed by a least-squares method, as previously reported [3]. In addition, the tentoxin-binding site was characterized by equilibrium ultrafiltration following experimental and analytical procedures reported earlier [3, 7].

The subunit composition of the digest was examined by electrophoresis in polyacrylamide gels containing SDS according to the methods of Laemmli [10]. Bovine serum albumin (M_r 69 000), glutamic dehydrogenase (E.C. 1.4.1.3; M_r 53 000), trypsin (E.C. 3.4.4.4; M_r 23 300) and cytochrome *c* (M_r 11 700) were used as markers.

RESULTS

The time course of Ca^{+2}-dependent ATPase activity during trypsin digestion

TABLE I

CHARACTERISTICS OF TENTOXIN BINDING TO TRYPSIN-TREATED LETTUCE CF_1

Modifying ligand(s)	Buffer[a]	Sites[b]	$K_a \cdot 10^{-8}$ M^{-1} [c]
None	Tris · HCl	0.60	3.86
Ca^{+2} (5 mM)	Tris · HCl	0.62	1.87
ATP (10 mM)	Tris · HCl	0.60	4.03
ADP (1 mM)	Tris · HCl	0.51	3.62
None	Tricine	0.55	1.51
Ca (2 mM)	Tricine	0.46	1.53
ATP (2 mM)	Tricine	0.60	3.82
ATP (2 mM), Ca (2 mM)	Tricine	0.63	3.06
ATP (2 mM), Ca (2 mM)	Tricine	–	2.3[d]

[a] Tris · HCl is 50 mM Tris · HCl, pH 8.0. Tricine is 20 mM Tricine/NaOH, pH 8.0.

[b] Mol of binding site/mol of CF_1, where mol CF_1 is calculated on the basis of M_r = 230 000.

[c] Affinity constant determined by equilibrium ultrafiltration [3, 7].

[d] Determined by steady state enzyme kinetics.

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

was similar to that reported by Deters et al. [6]. The specific activity increased from 1.6 to 6.7 μmol PO_4 min^{-1} mg^{-1} during the first hour of digestion. After 6 h, a maximum specific activity of 9.1 μmol PO_4 min^{-1} mg^{-1} was reached. Upon SDS gel electrophoresis of the digest, a single band was observed with an approximate molecular weight of 55 000. After prolonged destaining (3 weeks) of gels containing 50 μg of digest, two very faint bands of higher mobility (M_r 24 000 and 21 000) were visible; these may represent trace protein contaminants. Prior to trypsin digestion, lettuce CF_1 subjected to SDS electrophoresis exhibited five major protein bands corresponding to those of spinach CF_1.

Least-squares analysis of the ATPase-tentoxin inhibition curves yielded an apparent affinity constant of $2.3 \cdot 10^8$ M^{-1}. The results of the direct binding measurements, analyzed as a 1-ligand-1-site model without including competitive effects of ATP and ADP, are summarized in Table I. Consideration of these nucleotides as competitive ligands failed to improve the fit of the theoretical results to those obtained experimentally.

DISCUSSION

The extensively trypsinized lettuce CF_1 used in this investigation appears to consist predominantly of α and β subunits. Its behavior both upon SDS gel electrophoresis and during digestion is similar to that observed by Deters et al. [6] for spinach CF_1. As with the native enzyme, the tentoxin binding site of the digest has an affinity constant of $1 \cdot 10^8$–$4 \cdot 10^8$ M^{-1} and does not interact significantly with ATP or ADP. The apparent reduction in sites/mol of protein from 0.85 as previously reported [3] to 0.5–0.6 may be due to trypsin modification of the α and β subunits, as was noted by Deters et al. [6] and McEvoy and Lynn [11], or to the presence of extensively digested inactive protein. No tentoxin-insensitive ATPase appeared during digestion, and the observed sensitivity of the purified ATPase preparation paralleled that of the native enzyme.

These data taken together indicate that the tentoxin-binding site resides on the α and β subunit complex, and that this site is very probably the same as that observed in the undigested protein. Thus, tentoxin appears to inhibit the catalytic functions of CF_1 by binding to those subunits most closely associated with the catalytic site. This action is in contrast to that of the ε subunit which inhibits CF_1 through the mediation of the γ subunit [6].

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