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TENTOXIN

AN UNCOMPETITIVE INHIBITOR OF LETTUCE CHLOROPLAST COUPLING FACTOR 1

JOHN A. STEELE [a], RICHARD D. DURBIN [a], THOMAS F. UCHYTIL [a] and DANIEL H. RICH [b]

[a] *Plant Disease Resistance Research Unit, A.R.S., U.S.D.A., Department of Plant Pathology and* [b] *School of Pharmacy, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)*

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Summary

The interaction of tentoxin [*cyclo*(-L-leucyl-*N*-methyl-(Z)-dehydrophenylalanyl-glycyl-*N*-methyl-L-alanyl-)] with solubilized lettuce chloroplast coupling factor 1 was characterized by direct binding studies, measurement of the time course of ATPase inhibition, and steady-state enzyme kinetics. Neither substrates, products or Ca^{2+} competed with the tentoxin binding site, nor did they induce any large change in tentoxin affinity. The inhibition of lettuce chloroplast coupling factor 1 ATPase was found to be the time dependent, and at equilibrium the affinities estimated by equilibrium ultrafiltration and enzyme inhibition were similar ($1.8 \cdot 10^8$ M^{-1}). The steady-state kinetics best fit an uncompetitive pattern suggesting that the inhibited steps follow an irreversible step occurring after ATP binding.

Introduction

When the fungus *Alternaria alternata* (Fries) Keissler attacks cotton seedlings, a marked chlorosis results [1]. This effect is due to the presence of a toxic metabolite whose trivial name is tentoxin. On the basis of combined NMR, mass spectral and X-ray studies, Meyer et al. [2] assigned the structure of *cyclo*-(-L-leucyl-*N*-methyl-(Z)-dehydrophenylalanyl-glycyl-*N*-methyl-L-alanyl-) to this substance. This structure has recently been confirmed by chemical synthesis [3]. The purified toxin when applied to germinating seed of a wide range of plant species causes chlorosis in some species, but has little apparent effect on

Abbreviation: CF_1, chloroplast coupling factor 1.

others [4]. This differential response extends to the generic level in *Nicotiana* within which sensitive and insensitive species are known [5].

The selective chlorosis is due to the presence of a single tentoxin binding site on CF_1 of sensitive species [6]. When this site binds tentoxin, coupled electron transport in chloroplasts and the ATPase activity of solubilized CF_1 are inhibited. Insensitive species tested thus far lack such a binding site. However, other resistance mechanisms can be envisioned; e.g. exclusion of tentoxin from the chloroplast or metabolic alteration. Tentoxin is the only inhibitor of CF_1 or photophosphorylation reported to exhibit such selectivity. As such it is a potentially valuable tool for investigating the mechanisms of both photophosphorylation and plant disease resistance. As a prerequisite for such studies, the following report presents a more detailed characterization of tentoxin's interaction with CF_1.

Materials and Methods

Lettuce chloroplasts and CF_1, tentoxin, and [^{3}H]tentoxin were prepared as previously described [6]. Lettuce CF_1 was trypsin activated by the procedure of Lien and Racker [7]. In some cases, activation by trypsin was followed by additional electrophoretic purification. Polyacrylamide gels (7.5%) were prepared by the methods of Ornstein [8] and Davis [9], and 50—100 μg of trypsin-activated lettuce CF_1 was applied to each gel. The gels were run at room temperature at 3 mA/gel until the tracking dye emerged. They were then sliced into 0.5-mm segments and the slices collected in vials containing 1 ml of 40 mM tricine buffer (pH 8.0). After 3 h, the ATPase activity was located by enzyme assay and the active fractions pooled. Alternatively, 4% gels were used and the ATPase activity was located by the staining method of Horak and Hill [10]. This gel segment was then removed and minced into tricine buffer. Following these procedures, the final solution was brought to a protein concentration of 30—50 μg/ml using an Amicon B-15 Minicon.

The active site of lettuce CF_1 was reversibly inactivated by reaction with a 50% excess of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole using the method of Deters et al. [11] which selectively blocks tyrosine residues near the active site. The product was readily reactivated with 1 mM dithiothreitol.

Estimates of coupled electron transport rates in isolated lettuce chloroplasts were obtained from polarographic measurements of oxygen uptake using methods described by Steele et al. [6].

Assays for ATPase activity, unless otherwise stated, were done at 37°C in 1 ml of 40 mM tricine buffer (pH 8.0) containing various concentrations of ATP and $CaCl_2$. Inorganic phosphate was determined using the methods of Taussky and Schorr [12].

Enzyme kinetics data were analyzed by multiple linear regression methods or, in some cases, by iterative non-linear regression [13]. Affinity constants for tentoxin were determined by equilibrium ultrafiltration [6] employing lettuce CF_1 ($2 \cdot 10^{-7}$—$4 \cdot 10^{-7}$ M) and equivalent concentrations of [^{3}H]tentoxin (47 Ci/mol) in 4—6 ml of 20 mM tricine buffer (pH 8.0) containing various additions. Flow rates were maintained at 0.05 ml/min or less so that the protein-ligand equilibrium was not significantly perturbed. The experimental results

were analyzed using a combination of non-linear regression and simulation techniques which have been described in detail elsewhere [14]. The methods of Steele et al. [6], which account for inhibitor binding, were used when affinity constants were estimated from enzyme inhibition curves.

Results

The results of equilibrium ultrafiltration experiments performed under a variety of conditions are summarized in Table I. The binding of tentoxin was not detectably altered by the presence of Ca^{2+}, ATP, ADP or adenyl-5′-yl-imidophosphate. Blocking the tyrosines near the active site with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole had little or no effect, nor did activation by heat or trypsin greatly alter tentoxin binding.

The inhibition of CF_1 ATPase as a function of time when exposed to $4.8 \cdot 10^{-8}$ M tentoxin appears in Fig. 1. The estimated time to half-maximum inhibition is 5 min, but precise estimates cannot be obtained due to the necessity for a finite assay time.

To determine if steady-state concepts were applicable to the inhibition of CF_1 by tentoxin, trypsin-activated CF_1 (10 μg/assay) was incubated with 0, $2.4 \cdot 10^{-8}$, and $4.8 \cdot 10^{-8}$ M tentoxin for 2 h. The release of phosphate was followed at 1-min intervals from 3 to 10 min after addition of calcium and ATP. Over this interval the reaction rates were constant at each tentoxin concentration.

When trypsin-activated CF_1 was equilibrated with tentoxin for 2 h prior to ATPase measurement, the upper inhibition curve of Fig. 2 was obtained. The affinity constant estimated from this curve is $1.8 \cdot 10^8$ M^{-1}. A similar experiment employing the Tris/Mg^{2+}/bicarbonate buffer described by Nelson et al. [15] yielded an estimate of $1.6 \cdot 10^8$ M^{-1}.

TABLE I

INTERACTION OF TENTOXIN WITH LETTUCE CF_1 IN 20 mM TRICINE BUFFER (pH 8.0)

Protein treatment	Additions	$K_a \times 10^{-8}$ (M^{-1}) [a]	Sites [b]
Native	None	2.0	0.81
Native	Ca^{2+} (10 mM)	2.2	0.84
7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole [c]	Ca^{2+} (5 mM)	3.2	0.68
Heat activated [d]	Ca^{2+} (5 mM)	3.1	0.85
Trypsin activated [e]	None	1.8	0.55
Trypsin activated [e]	Ca^{2+} (10 mM)	3.0	0.62
Trypsin activated [e]	Ca^{2+} (10 mM) + ADP (10 mM)	1.7	0.57
Trypsin activated [e]	Ca^{2+} (10 mM) + ATP (10 mM)	2.3	0.70
Trypsin activated [e]	PO_4^{3-} (10 mM)	3.0	0.74

[a] Affinity constants determined by equilibrium ultrafiltration [14].

[b] Mol of binding site/mol of CF_1 where mol of CF_1 is calculated on the basis of 325 000 molecular weight.

[c] Treated with 1.5 equivalents of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole according to the methods of Deters et al. [11].

[d] Heat activated according to Lien and Racker [7].

[e] Mild trypsin digestion according to Lien and Racker [7].

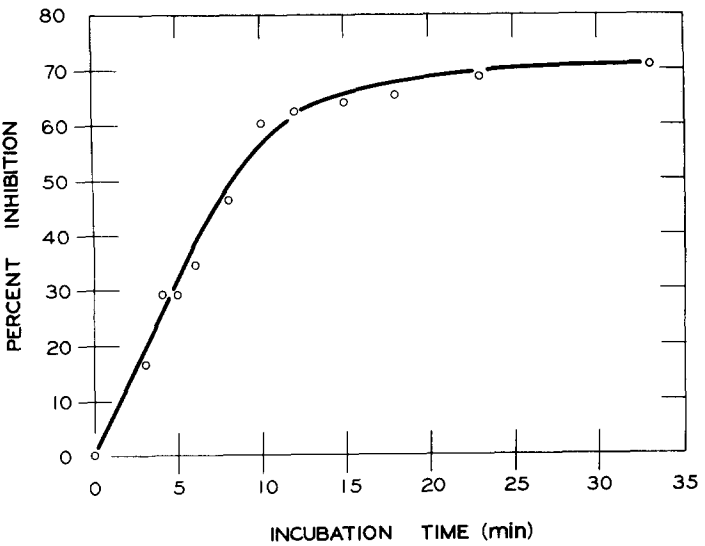

Fig. 1. A plot of the percent inhibition of trypsin-activated lettuce CF_1 ATPase activity vs. time at a tentoxin concentration of $4.8 \cdot 10^{-8}$ M. The assays were carried out in 1.0 ml of 40 mM tricine buffer (pH 8.0) in the presence of 2 mM $CaCl_2$, 2 mM ATP, and 15 μg protein. The reaction was initiated by the addition of ATP and $CaCl_2$ following the incubation of the enzyme with tentoxin. After 3 min at 37°C the reaction was quenched with 1 ml of 0.5 M trichloroacetic acid and inorganic phosphate was determined. The times plotted are the sum of the preincubation period and the 3 min required for assay.

The effect of tentoxin on CF_1 activity prior to equilibration was determined by measuring ATPase activity after 5 min of exposure to a range of tentoxin concentrations. Under the conditions of the experiment, inhibition at a fixed

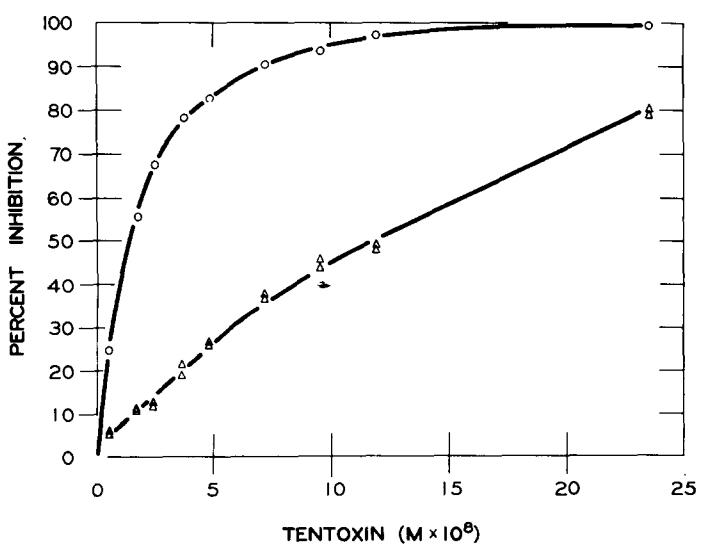

Fig. 2. Plots of percent inhibition of trypsin-activated lettuce CF_1 ATPase vs. tentoxin concentration at equilibrium (○) and disequilibrium (△). The assay mixture contained 2 mM $CaCl_2$, 2 mM ATP, 10 μg protein and a variable tentoxin concentration. Assays were terminated after 5 min at 37°C. The equilibrium curve was obtained by equilibrating CF_1 and tentoxin for 2 h and initiating the reaction with Ca^{2+} and ATP. The curve before equilibrium had been reached was obtained from assays initiated by the addition of CF_1.

time was very nearly linear with respect to tentoxin even at concentrations that would saturate the binding site at equilibrium (Fig. 2).

The results obtained using trypsin-activated CF_1 purified on 7.5% polyacrylamide gels and ATP concentrations of 1—4 mM are presented in Fig. 3. Results of a similar experiment employing trypsin-activated CF_1 and a range of ATP from 0.25 to 2 mM appear in Fig. 4.

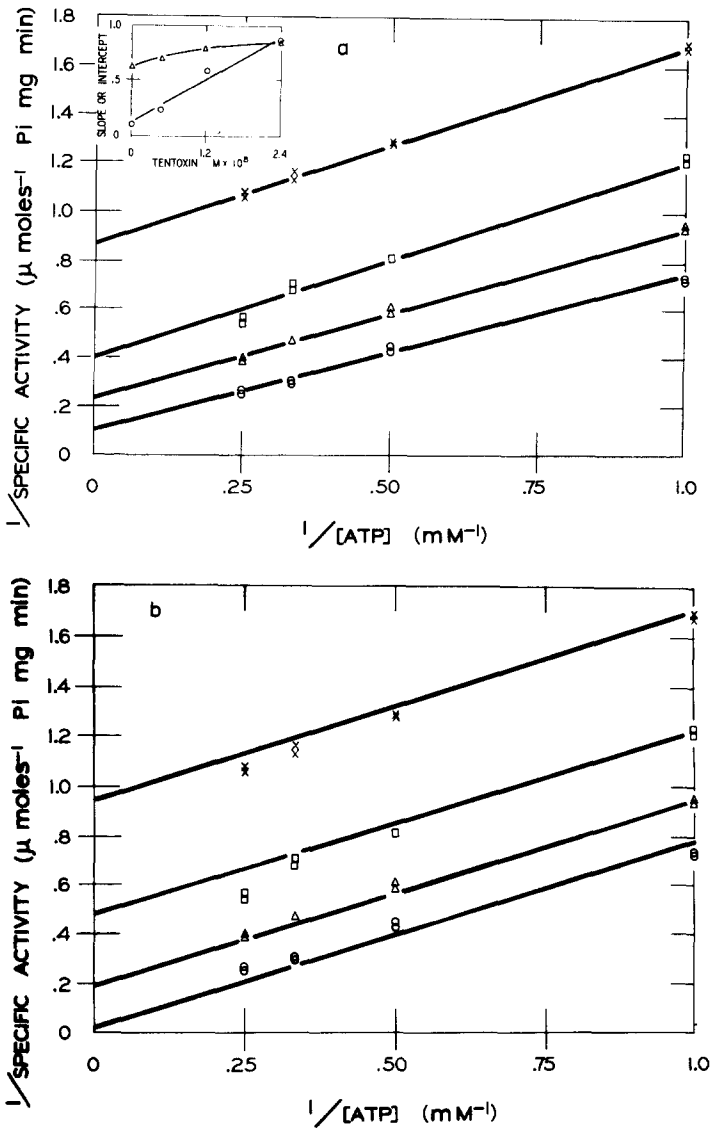

Fig. 3. Double reciprocal plots of ATPase activity of electrophoretically purified, trypsin-activated lettuce CF_1 at various tentoxin concentrations. Assays were carried out in the presence of 10 mM $CaCl_2$, 1—4 mM ATP, $0—24 \cdot 10^{-8}$ M tentoxin and 3 μg of protein. All components except $CaCl_2$ and ATP were equilibrated for 2 h and the reactions were initiated by adding $CaCl_2$ and ATP. After 30 min at 37°C the reaction was terminated and phosphate was determined. The plotted lines are fitted (a) individually by linear least squares or (b) by multiple regression to a linear uncompetitive model. The tentoxia concentrations were 0 (○), $4.8 \cdot 10^{-9}$ M (△), $12 \cdot 10^{-9}$ M (□), and $24 \cdot 10^{-9}$ M (X). The inset is a plot of the intercepts (○) and slopes (△) of the individually fitted lines with respect to tentoxin concentration.

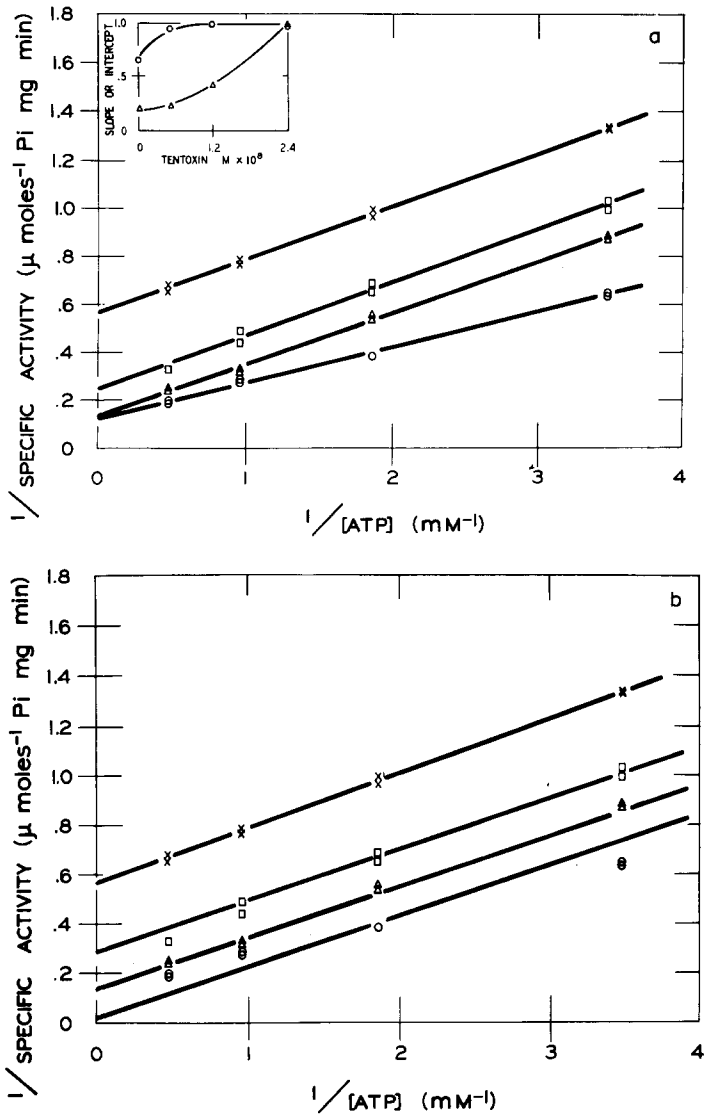

Fig. 4. Double reciprocal plots of specific activity vs. [ATP] at various tentoxin concentrations. Assays were carried out as in Fig. 3 but over a lower range of ATP concentration. The lines are fitted (A) individually by linear least squares or (B) by multiple regression to a linear uncompetitive model. The tentoxin concentrations were 0 (○), $4.8 \cdot 10^{-8}$ M (△), $12 \cdot 10^{-8}$ M (□) and $24 \cdot 10^{-8}$ M (X). The inset is a plot of the intercepts (○) and slopes (△) of the individually fitted lines with respect to tentoxin concentration.

The estimated kinetic parameters for uncompetitive, competitive, and mixed inhibition systems are shown in Table II. In addition, estimates from lines fitted by least squares at each tentoxin concentration are tabulated. Both the uncompetitive and mixed models approximate the experimental results reasonably well while the competitive model provides a distinctly inferior fit. When the data for individual tentoxin concentrations are fitted by linear least squares (Figs. 3A and 4A) there is no apparent bias and the replicated measurements at each ATP concentration indicate that the amount of pure error is small. Re-

TABLE II

LINEAR REGRESSION ESTIMATES OF KINETIC PARAMETERS FOR DATA PRESENTED IN FIG. 3 AND 4 FITTED TO VARIOUS MODELS

Parameter	Model							
	Individually fitted		Uncompetitive [c]		Competitive [d]		Mixed [e]	
	a	b	a	b	a	b	a	b
V (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	10.0	8.0	79	95	2.5	4.0	10	18 [f]
K_s (mM)	6.7	1.3	59	20	33	43	6.0	3.3
K_a ($M^{-1} \times 10^{-8}$)	2.3 [i]	1.3 [i]	7.5	7.0	4.1	1.8	6.3	100
α [j]	—[g]	—[g]	—[g]	—[g]	—[g]	—[g]	0.09	0.03
F [k]	173 [h]	191 [h]	566	510	112	121	621	441
r^2 [l]	0.97 [h]	0.98 [h]	0.97	0.97	0.88	0.89	0.98	0.97
df [m]	7	7	28	28	28	28	27	27

[a] Data from Fig. 3.
[b] Data from Fig. 4.
[c] $1/v = (K_s/V)*1/[S] + (K_a/V)*[I] + 1/V$ [19].
[d] $1/v = (K_s/V)*1/[S] + (K_s*K_a)V)*[I]*1/[S] + 1/V$ [19].
[e] $1/v = (K_s/V)*1/[S] + (K_a/\alpha V)*[I] + ((K_a*K_s)/V)*[I]*1/[S] + 1/V$ [19].
[f] Standard error of estimate greater than 25% of estimated value.
[g] Not applicable.
[h] Average at 7 degrees of freedom.
[i] At saturation with ATP.
[j] α = Constant defined in ref. 19.
[k] F = F statistic for regression.
[l] r^2 = Coefficient of determination.
[m] df = Degrees of freedom for regression.

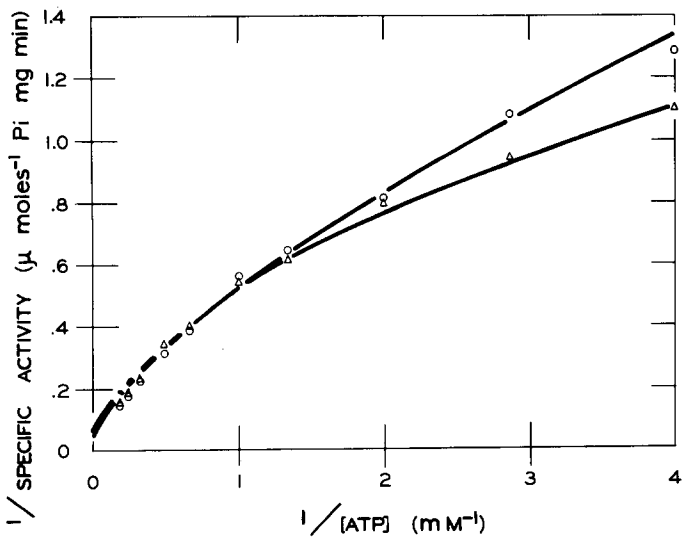

Fig. 5. Double reciprocal plots of specific activity vs. [ATP] for trypsin-treated, electrophoretically purified lettuce CF_1 (○) and the same preparation prior to trypsin treatment (△). The curves were fitted by non-linear regression to the model $1/V = 1/((V*[ATP])/(K_m + [ATP]) + (V'*[ATP]/(K'_m + [ATP]))$ where V and K_m are the maximum velocity and Michaelis constant for one enzyme form and V' and K'_m are the equivalent values for the second component, respectively.

plots of slopes and intercepts indicate a slight non-linear dependence of slope upon tentoxin concentration for the electrophoretically purified, trypsin-activated CF_1 (Fig. 3A) and a more pronounced effect when this final purification was omitted (Fig. 4A). The abrupt change of slope with the less purified material introduces bias into the uncompetitive model in the region of the tentoxin-free treatment that exceeds the level of pure error (Fig. 4B); the mixed model is similarly biased. In the case of electrophoretically purified CF_1, this bias is considerably reduced (Fig. 3B). The data of Fig. 4 were also examined as a plot of $I/(1-V_i/V_o)$ vs. V_o/V_i as described by Henderson [16] for tight binding inhibitors. The results fit an uncompetitive pattern.

The biases present in these results led us to examine the behavior of both native and trypsin-treated, electrophoretically purified CF_1 over a wide range of ATP concentrations (Fig. 5). The responses are non-linear and trypsin treatment followed by electrophoretic purification reduces, but does not eliminate, the non-linearities.

Gel electrophoresis on 4% acrylamide followed by staining for ATPase revealed a single ATPase band in the native protein and three active bands in the trypsinized preparation prior to its purification (Fig. 6).

The kinetics of ATP synthesis in isolated lettuce chloroplasts as measured by couplied electron transport appear in Figs. 7. The particulate nature of the coupled chloroplast assay and the presence of a basal transport rate preclude obtaining precise data over a wide range of substrate and inhibitor concentrations. However, our results appear to be uncompetitive over the ADP concentrations tested.

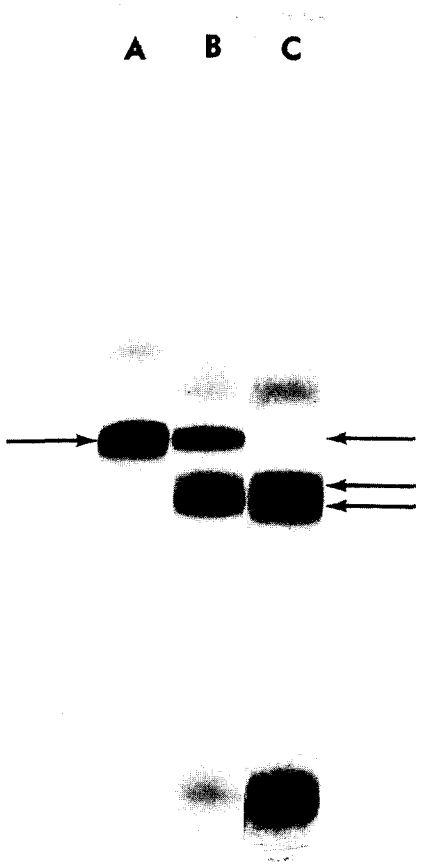

Fig. 6. Lettuce CF_1 run in 4% polyacrylamide gels before and after trypsin treatment according to Lien and Racker [7]. The samples are (a) CF_1, (b) CF_1 mixed with trypsin-treated CF_1, and (c) trypsin-treated CF_1. The Ca^{2+}-dependent ATPase bands as determined by the method of Horak and Hill [10] are marked with arrows.

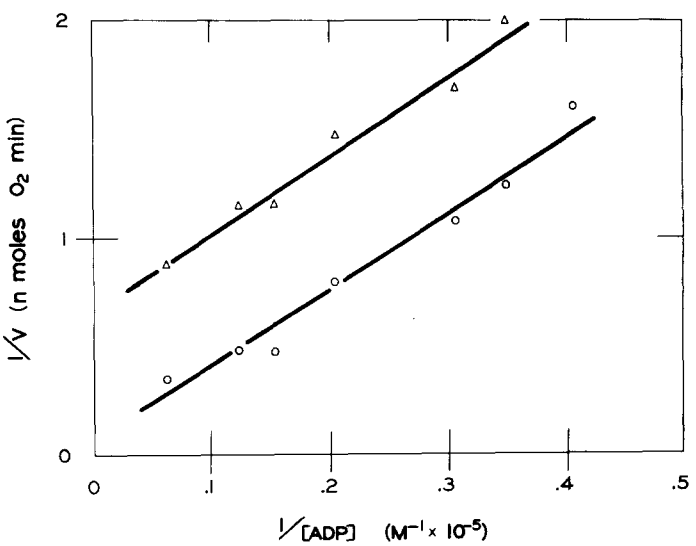

Fig. 7. Double reciprocal plots of coupled electron transport rates of lettuce chloroplasts vs. ADP concentration with (△) and without (○) tentoxin ($2.5 \cdot 10^{-7}$ M). Chloroplasts containing 1 mg of chlorophyll were equilibrated with the indicated concentrations of ADP and tentoxin for 2 h in 1 ml of 40 ml tricine buffer (pH 8.0) containing 0.3 M sucrose. Assays (50 mg chlorophyll) were done and corrected for basal transport rates as described by Steele et al. [6]. The lines are individually fitted by linear least squares.

Discussion

The observed slow interaction of CF_1 with tentoxin accounts for the disparity reported earlier [6] between affinity constants estimated from enzyme inhibition data ($K_a = 1.3 \cdot 10^7\ M^{-1}$) and direct binding measurements ($K_a = 2 \cdot 10^8\ M^{-1}$). When this slow interaction is allowed to proceed to near equilibrium, enzyme inhibition data yields a K_a of almost $2 \cdot 10^8\ M^{-1}$ in either the magnesium- or calcium-activated systems, suggesting that the tentoxin site is not affected by the identity of the activating ion and that the original estimates of K_a obtained by equilibrium ultrafiltration are correct.

The enzyme inhibition patterns are most readily interpreted as being uncompetitive with respect to ATP and ADP. This conclusion is strengthened by the observation that the nucleotide substrates, phosphate, and calcium separately or in combination neither compete at the tentoxin site nor greatly alter the tentoxin affinity constant.

The slight, but real, deviation from a purely uncompetitive system could arise from the non-linearities observed in double reciprocal plots over a wide range of ATP concentrations. The observed electrophoretic heterogeneity of trypsin-activated CF_1 (Fig. 6), and the reduced deviation encountered after electrophoretic purification indicate that these non-linearities might arise from multiple enzyme forms. Among other possible causes of such non-linearities are: (1) the complex kinetics of metal-activated enzymes [17]; (2) the slow association of CF_1 with nucleotides [18]; and (3) the allosteric control exerted by ADP [18]. These factors might individually or collectively cause the observed deviations.

If the inhibition patterns are uncompetitive, tentoxin interferes with steps following ATP binding in the case of solubilized CF_1, and steps following ADP binding in the membrane-bound system. Since tentoxin binds to many of the enzyme forms, a non-competitive or mixed inhibition would be expected. However, an uncompetitive pattern would result if: (1) the inhibited steps occur after an irreversible sequence that is preceded by ATP binding; and (2) the functionality of those enzyme forms preceding this sequence are unaffected. The inhibited events may include the catalytic steps of phosphate transfer. Tentoxin thus might be a useful compound in studies of the enzymatic mechanism of CF_1. In addition, since CF_1 can be successfully isolated from many species (Durbin, R.D., unpublished), reconstitution experiments employing chloroplasts and CF_1 from species exhibiting differential sensitivities may be possible. In the presence of tentoxin the results of such experiments should be unambiguous.

Acknowledgements

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