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PURIFICATION AND CHARACTERIZATION OF 3-DEHYDROQUINATE HYDROLASE AND SHIKIMATE OXIDOREDUCTASE

EVIDENCE FOR A BIFUNCTIONAL ENZYME

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Summary

The basis for the physical association of 3-dehydroquinate dehydratase (3-dehydroquinate hydrolyase, EC 4.2.1.10) and shikimate dehydrogenase (shikimate:$NADP^+$ 3-oxidoreductase, EC 1.1.1.25) in higher plants was investigated. The enzymes were extracted from the moss *Physcomitrella patens* and were purified to homogeneity. Determinations of subunit sizes were made by sodium dodecyl sulfate gel electrophoresis and gel exclusion chromatography in 6 M guanidinium chloride. Results from these studies demonstrate that both enzyme activities are carried out by a single polypeptide.

Introduction

In *Neurospora crassa*, the five enzymes catalyzing steps 2—6 in the prechorismate polyaromatic amino acid biosynthetic pathway (the so called shikimate pathway) are physically associated as a functional enzyme aggregate [1]. The genes encoding these five enzymes have been shown to map as a 5 gene cluster [2]. By contrast most prokaryotes lack any enzymic aggregation [3], and the genes encoding the enzymes do not map as a cluster [4—6]. In eukaryotic plants, however, two of the enzymes, 3-dehydroquinate dehydratase (3-dehydroquinate hydrolyase, EC 4.2.1.10) and shikimate dehydrogenase (shikimate: $NADP^+$ 3-oxidoreductase, EC 1.1.1.25) have activities that are always found associated even after various procedures are employed in an attempt to separate them [7—9]. The other three enzymes which form an aggregate in *Neurospora* (5-dehydroquinate synthetase, shikimic acid kinase, and 3-*enol*pyruvalshikimate 5-phosphate synthetase) are clearly separable in plants by sucrose density centrifugation.

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The nature of the association of the hydrolyase and the reductase, its genetic basis and the functional role that the associated enzymes play in plants are considered in this report. Although information regarding the organization of the genes encoding for the hydrolyase and the reductase has not yet been obtained, information from the biochemical studies reported here provides conclusive evidence regarding the nature of their association, as well as offering insight into possible functional roles for this association in plants.

Materials and Methods

Culture of Physcomitrella patens. Sterile cultures of *P. patens* have been maintained in this laboratory and were originally derived from vegetatively propagated subcultures of a culture grown from a single spore [10]. Stock cultures were maintained on agar slants. Mass cultures of the moss were grown in a Brunswick Table Top Microfermentor that was fitted with a bank of fluorescent lights. Approx. 1 g fresh weight of moss protonemata that had been growing in small liquid culture was added to 12 l sterile Hutner's medium [11]. Compressed air plus 2.5% CO_2 was bubbled through the medium at 1 l/min per l medium. The light source was provided by Naturescent Lamps at an intensity of about 1000 lux. The cultures were allowed to grow at 27°C for 6 days; at this time cultures were collected by filtration and the cells were washed with 2 l 0.1 M phosphate buffer (pH 7.5, 4°C). The packed cells were then stored at −70°C until used.

Enzyme assays. The hydrolase and the reductase activities were measured during all stages of purification by modifications of published procedures [2]. The activity of the hydrolyase was followed by measuring the dehydration of 3-dehydroquinate to 3-dehydroshikimate at 240 nm. The reaction mixture contained 10 mM dehydroquinate in 0.1 M potassium phosphate buffer (pH 7.5). 10 µl enzyme were added to 1 ml reaction mixture and the increase in A_{240nm} was measured at 35°C.

The reductase was measured by following the increase in absorbance at 340 nm (35°C) during the oxidation of shikimic acid to 3-dehydroshikimic acid (NADP reduction). The reaction mixture contained 50 mM shikimic acid, 1.0 mM NADP in 0.05 M glycine/KOH buffer (pH 10.6) and 10 µl enzyme solution were added.

DEAE-cellulose chromatography. DEAE-cellulose (DE-52) was prepared for use by equilibrating in 0.05 M potassium phosphate buffer (pH 7.5), 0.5 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. The enzyme preparation was eluted from a 2.5 × 100 cm column by a 1 l KCl gradient (0.05—0.35 M) at a flow rate of 32 ml/h.

Hydroxyapatite chromatography. The hydroxyapatite was obtained as a pre-swollen gel and was equilibrated with above phosphate buffer at pH 6.9. The enzymes were eluted from a 1.5 × 40 cm colum by a linear gradient of 0.05—0.2 M potassium phosphate buffer (pH 6.9).

Cibracon blue F3GA-Sepharose 4B chromatography. The procedure for attaching the dye Cibracon blue (Ciba/Geigy) to Sepharose was adated from the procedure of Böhme et al. [12] for attaching the dye to Sephadex G-200. The former medium resulted in faster flow rates. The enzymes were eluted from a

2.5 × 100 cm column by a linear gradient of 0.05—0.3 M potassium phosphate buffer (pH 7.5).

Sucrose density centrifugation. The procedure employed was adapted from Martin and Ames [13]. Linear gradients of 5.5—20% sucrose were prepared and allowed to stand at 4°C prior to use. Centrifugation was carried out on a Beckman SW 65 rotor at 40 000 rev./min for 20 h. 10-drop fractions were collected and assayed for enzyme activity. Linearity was checked by measuring the refractive index of the fractions.

GuHCl gel exclusion chromatography and sodium dodecyl sulfate (SDS) gel electrophoresis. The procedure for gel exclusion chromatography in the presence of 6 M guanidium chloride (GuHCl) was that of Fish and Mann [14]. Purified enzyme was eluted from a 0.9 × 140 cm jacketed column heated to 55°C at a hydrostatic pressure of 20 cm and a flow rate of 3 ml/h. 1-ml fractions were collected and assayed for protein at 220 nm or by the method of Schaffner and Weismann [15].

The method of gel electrophoresis in the presence of sodium lauryl sulfate employed was that of Weber and Osborn [16].

Protein determinations. Protein concentrations were measured by either the procedure of Lowry et al. [17] or that of Schaffner and Weismann [15].

Results

Inseparability of the hydrolase and the reductase

Crude extracts obtained from 50—75% $(NH_4)_2SO_4$ fractionation of total soluble protein were subjected to ion-exchange and gel exclusion chromatography. It was found that the hydrolyase and the reductase co-eluted from DEAE-cellulose, hydroxyapatite and Sephadex G-200. Similar results were reported earlier [8,9]. Another method, chromatography with Sepharose 4B modified by the attachment of Cibracon blue, has not been used previously and was employed in an effort to separate these two enzyme activities. The profile of enzyme elution is shown in Fig. 1. The double peak is characteristic of these two enzymes when chromatographed on this resin. It is significant to note that

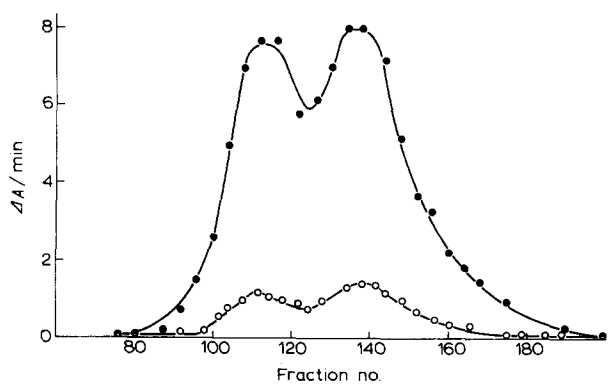

Fig. 1. Cibracon blue F3GA-Sepharose 4B chromatography. Shikimate oxidoreducatse activity (•) measured at 340 nm and 3-dehydroquinate hydrolyase activity (○) measured at 240 nm.

both enzymes had this profile. The basis for this particular behavior is not clearly understood. The fact that the double peak may represent a contaminating species or an isozyme seems unlikely since polyacrylamide gel electrophoresis of each peak, run separately and as a mixture gave only one band. Furthermore, when either peak was rerun on similar columns, the double peak elution profile was retained.

Similar co-elution for these enzymes extracted from a variety of plants was observed previously. These findings and those reported above suggest a physical association of the two enzymes. The basis of this association comes from the characterization of physical and molecular properties of purified enzyme complex.

Purification of the hydrolyase and the reductase

Approx. 25 g lyophilized moss protonema (400 g fresh weight) were ground in a Wiley mill. 30 ml 0.1 M potassium phosphate buffer (0.5 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride, pH 7.5) were added for each gram dry weight of moss. This mixture was stirred gently for 45 min at 4°C; all subsequent steps were also preformed at this temperature. Cellular debris was removed by centrifugation for 15 min at 13 000 $\times g$ and the supernatant was decanted. The nucleic acids were precipitated by the addition of 1/10 volume 1.4% protamine sulfate solution and were removed by centrifugation. Proteins were precipitated by 50—75% $(NH_4)_2SO_4$. The resulting protein pellet was redissolved into 1/10 volume extraction buffer, and the redissolved protein dialyzed overnight against two changes of 0.05 M potassium phosphate buffer (pH 7.5)/0.5 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride.

After dialysis, the protein solution was loaded onto a column of DEAE-cellulose and eluted by a linear KCl gradient. Fractions containing the enzyme activities were pooled and concentrated in an Amicon concentrator under 20 lbs/inch^2 N_2. The concentrated protein was dialyzed for 12 h against 0.05 M phosphate buffer (pH 7.5) and then loaded onto a Cibracon blue-Sepharose 4B column and eluted. Active fractions were pooled, concentrated and prepared for hydroxyapatite chromatography by dialyzing against 0.05 M phosphate buffer (pH 6.9) for 12 h. The enzymes were eluted by a linear phosphate buffer gradient. The fractions containing enzyme activities were divided into three and were analyzed for purity by polyacrylamide gel electrophoresis at pH 7.0 and 8.9. The center fractions were usually pure at this stage. Any contaminating protein that might be present was removed by repeating the hydroxyapatite chromatography; generally, this was not necessary. Table I summarizes a typical purification.

Determination of the molecular weight

The Stokes radius of the hydrolyase-reductase complex was measured at 31 Å by Sephadex G-200 chromatography [18]. The sedimentation coefficient was determined by sucrose density centrifugation [13] as shown in Fig. 2. The s value obtained for this enzyme complex equals $3.8 \cdot 10^{-13}$ S. The average partial specific volume of 0.725 for proteins was used in this calculation. A calculated molecular weight of 49 500 was obtained.

TABLE I

PURIFICATION OFHYDROLASE AND REDUCTASE

Purification steps	Protein (mg)	Reductase (μmol product/min)	Hydrolase (μmol product/min)	Fold purification		Specific activity	
				Reductase	Hydrolase	Reductase	Hydrolase
Crude	3685	432	125	1	1	0.12	0.034
Protamine SO_4	3600	488	129	—	—	0.14	0.04
50—75% $(NH_4)_2SO_4$	900	427	112	4	4	0.47	0.12
DEAE-cellulose	223	347	105	13	14	1.56	0.47
Cibracon blue-Sepharose 4 B							
I *		36	10				
II	2.2	75	23	283	294	34	10
III		36	12				
Total		148	85				
Hydroxyapatite							
I							
II	0.2	32	9	1333	1330	160	45
III							
Total							

* Fractions with activity were divided into thirds.

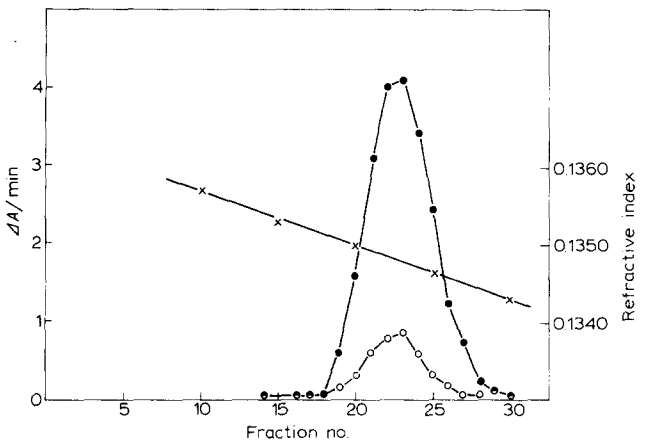

Fig. 2. Sucrose density centrifugation. Shikimate oxidoreductase activity (●) measured at 340 nm and 3-dehydroquinate hydrolyase activity (○) measured at 240 nm. Refractive index of fractions is designated by X's.

Subunit composition of the hydrolyase-reductase enzyme complex

The purified complex was subjected to SDS gel electrophoresis [16]. The relative migration of standard proteins and the hydrolyase-reductase complex were measured and plotted as a function of their molecular weights. The molecular weight of the peptide of the smallest peptide subunit of the enzyme complex was calculated at 48 000 which is the same size as the native complex.

In order to confirm this result, another procedure was employed. Gel exclusion chromatography in the presence of 6 M GuHCl at 55°C was used to calculate the molecular of peptides [14]. By this procedure, the molecular weight of the subunits of the complex was calculated to be about 43 500.

Both of these procedures failed to demonstrate a subunit peptide smaller than the native complex. The conclusion is that the complex is in fact a single polypeptide capable of catalyzing both reactions.

Discussion

A procedure for extracting and purifying the presumed enzyme complex (3-dehydroquinate hydrolyase, EC 4.2.1.10 and 3-dehydroshikimate oxidoreductase, EC 1.1.1.25) was developed. A very high degree of purification, approx. 1300-fold was achieved by the procedure described in the text. An important aspect of the purification of these two enzyme activities is that the ratio of their activities remains fairly constant throughout the purification. This fact alone argues convincingly against the notion that the association of the hydrolyase and the reductase is merely coincidental. Although the yield of pure enzyme complex afforded by this procedure is low, it provided sufficient material for a characterization of some physical properties. A Stokes radius of 31 Å and a sedimentation coefficient of $3.8 \cdot 10^{-13}$ S were calculated for the enzyme complex. From these values, the native molecular weight of 49 000 was calculated.

The most interesting discovery in these studies is that the smallest subunit of

the enzyme complex is approx. 48 000, which is identical to the molecular weight of the native complex. It is obvious then, that the description of hydrolyase and reductase as an enzyme complex is less than adequate. More accurate is the statement that these activities comprise a bifunctional enzyme consisting of a single polypeptide. Clearly, a single polypeptide comprising a bifunctional enzyme is not common but it is not without precedent (e.g. ref. 19).

The functional siginificance of the *Neurospora* aggregate is attributed to the channeling of intermediates, particularly dehydroquinate and dehydroshikimate which are potentially competed for by the inducible quinic acid catabolic pathway [3,20], the significance of the bifunctional enzyme in higher plants and the moss in particular is less clear. The difficulty in defining a clear functional role of the bifunctional enzyme arises in part from the inability to demonstrate clearly the presence of a quinate catabolic pathway in the moss. Although the enzymes of the quinate pathway undoubtedly exist because of the ubiquitous occurrence of quinic acid in plants, those enzymes that have been extracted from various plant tissue have proved to be extremely labile [21,22]. The lability of these enzymes may explain the inability to obtain unequivocal evidence of their presence in *P. patens.*

A common characteristic of organisms possessing both the *arom* aggregate and the catabolic pathway is the existence of a second, often inducible, dehydroquinate hydrolyase that is distinguishable genetically and physically from the biosynthetic dehydroquinate hydrolyase. It is the presence of this catabolic hydrolyase, along with the other catabolic enzymes that permits the conversion of quinic acid to protocatechuic acid without the need for any of the biosynthetic enzymes. Thus it is the hydrolyase "isozyme" that provides the crux of the channeling hypothesis proposed for the *arom* aggregate. However, at no time during the extraction or purification of the hydrolyase-reductase enzyme was there any evidence for the presence of an isozyme of hydrolyase in *P. patens.* Attempts to induce the synthesis of a second hydrolyase were unsuccessful although there is evidence that exogenously supplied quinate or shikimate will induce the activity of hydrolyase 4-fold in mung bean seedlings [23]. Recently Boudet et al. [24] reported the presence of a second hydrolyase free of the hydrolyase activity associated with the reductase. This enzyme is unlike its counterpart in *Neurospora* in that it is constitutive and is extremely heat labile.

The existence of a second hydrolyase in corn lends support to a channeling of intermediates as the function of the bifunctional enzyme. A precise understanding of the regulation and expression of the catabolic enzymes awaits further study.

After this work was completed, Koshiba [25] reported the purification of the hydrolyase-reductase enzyme from *Phaseolus.* It is of interest to note that similar specific activities for the purified enzyme are obtained. Koshiba [25] also reports the existence of an isozyme of the enzyme complex as determined by gel electrophoresis. At no time was an isozyme observed during the electrophoresis of proteins from moss extracts. The double peak obtained during Cibracon blue-Sepharose 4B chromatography is unlikely to be caused by the elution of isozymes since either peak rechromatographed alone demonstrated the double peak elution profile. There are several possible explanations for

Koshiba's results. For example, seeds are comprised of different tissues and one or the other isozyme may be tissue specific and reflect a differentiated state. The moss tissue used in these experiments was comprised of one cell type. In any event the report of this isozyme is of interest and its role in the regulation of the shikimic acid pathway awaits further study.

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