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PROTEOLYTIC INACTIVATION OF A PENTAFUNCTIONAL ENZYME CONJUGATE: COORDINATE PROTECTION BY THE FIRST SUBSTRATE[1]

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Summary. The arom pentafunctional enzyme conjugate of Neurospora crassa was exposed to trypsin, chymotrypsin, or a protease preparation from Neurospora in the presence and absence of the first substrate, 3-deoxy-D-arabino-heptulosonate 7-phosphate. It was found that the first substrate coordinately protects all five activities from proteolytic inactivation, which indicates a conformational change induced by this compound. In addition, the data presented are consistent with the "domain" theory of conjugate structure. It is also argued that coordinate protection may be of physiological significance.

INTRODUCTION

The arom enzyme system of Neurospora crassa is a pentafunctional enzyme conjugate, in which all five enzymes exist on a single polypeptide chain (1, 2). The enzyme system catalyzes a sequence of five steps in the synthesis of the aromatic amino acids (Fig. 1). Recently it was shown that the first substrate of the arom conjugate, DAHP,[3] coordinately activates four of the five activities of this multifunctional protein (3). Here we show that all five activities are protected against proteolytic inactivation when the first substrate is present.

MATERIALS AND METHODS

Preparation of enzyme. The arom enzyme conjugate was purified to homogeneity, free of proteolytic clips (determined by detergent gel electrophoresis), from an early log-phase culture of N. crassa, as previously described (1) with some modifications (Cole, Vitto, and Gaertner, in preparation).

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[3] Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate.

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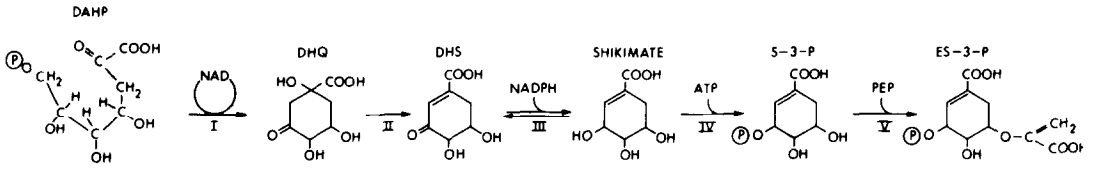

Fig. 1. The arom pathway of N. crassa. The five enzymes are: I, DHQ synthase; II, dehydroquinase; III, DHS reductase; IV, shikimate kinase; V, ES-3-P synthase. DHQ = dehydroquinate; DHS = dehydroshikimate; S-3-P = shikimate 3-phosphate; ES-3-P = 5-enolpyruvylshikimate 3-phosphate.

Substrates. DAHP and all substrates required for enzyme assays were prepared or purchased as previously described (3).

Enzyme assays. The five activities of the arom conjugate were assayed by methods described earlier (3). Proteolytic activity was determined by the Azocoll procedure (4). N. crassa protease activity is expressed in Azocoll units (one absorbance unit at 520 nm per 100 min).

Inactivation studies. Purified arom conjugate at a final concentration of 0.12 international units dehydroshikimate reductase/ml was incubated in 0.1 M potassium phosphate buffer (pH 7.0), alone or with various amounts of trypsin, chymotrypsin, or a protease preparation from N. crassa, at 25°C. Various concentrations of DAHP were also included in some of the incubation mixtures. Each enzyme of the conjugate was assayed at various times during the incubation period.

Proteases. Trypsin and chymotrypsin were obtained from Sigma. Trypsin (bovine pancreas, type III) was designated by the manufacturer as containing 12,000 BAEE units/mg. Chymotrypsin (bovine pancreas, type I-S) was designated as containing 40 BTEE units/mg. Trypsin activity, as determined by the hydrolysis of Azocoll (4), was 3700 units/mg, and chymotrypsin activity was 140 units/mg. The protease preparation from N. crassa was obtained as described previously (4); its activity, determined by the Azocoll method, was 180 units/ml.

RESULTS

All five enzymes of the conjugate are protected from proteolytic inactivation to varying degrees by DAHP. Some activities are completely stabilized, and, with one exception (the trypsin inactivation of the first enzyme), 10 mM DAHP (the highest concentration used) does not appear to saturate the system. Although the data are not presented in Table I, protection is seen with concentrations of DAHP as low as 50 μM or lower when protease levels are reduced. Hence, a protection appears to become more effective as DAHP concentrations are increased and protease levels are reduced. The marked protection afforded shikimate kinase by 5 and 10 mM DAHP against inactivation by the N. crassa protease preparation is noteworthy.

TABLE I. Effects of DAHP on the Time Required for Three Protease Preparations to Inactivate the Initial Activities of the Arom Enzyme Conjugate by 50%

Protease*	Enzyme†	50% Inactivation time (min) for DAHP concentrations of:			
		No DAHP	1 mM	5 mM	10 mM
Trypsin, μg/ml					
0.67	IV	26	34	44	69
2.5	I	46	149	156	156
50.0	III	28	44	68	90
100.0	V	42	82	134	200
100.0	II	46	106	240	356
Chymotrypsin, μg/ml					
0.67	IV	30	40	56	84
50.0	I	36	98	110	138
1000.0	III	40	91	(60%)‡,§	(75%)‡,§
1000.0	V	52	78	118	182
2000.0	II	64	(63%)‡	(81%)‡	(100%)‡,§
N. crassa protease, units/ml					
1.8	IV	54	88	(86.5%)‡	(90.5%)‡
18.0	I	28	88	110	138
18.0	III	90	172	220	272
18.0	V	122	182	264	320
18.0	II	106	194	300	350

*From initial experiments, an attempt was made to choose a protease concentration which would inactivate the enzymes in 30 min to 1 hr. This goal was accomplished in each instance except for the most stable enzymes and the N. crassa protease preparation.

†I, dehydroquinate synthase; II, dehydroquinase; III, dehydroshikimate reductase; IV, shikimate kinase; V, 5-enolpyruvylshikimate 3-phosphate synthase.

‡Percent activity remaining after 6 hr.

§Activity stabilized (i.e., no loss of activity observed once this percent of initial activity is reached).

In the absence of DAHP, enzymes II, III, and V have similar stabilities and are all relatively resistant to proteolytic inactivation. (Note the higher levels of proteolytic activity needed to accomplish the same or similar rate of inactivation.) The first enzyme in the pathway is much more sensitive to proteolysis; however, the fourth enzyme is by far the most sensitive.

DISCUSSION

These data lend further support to the idea that the organization of several enzymes on a single polypeptide chain may confer upon the overall system properties which an unorganized system would not normally be expected to have. The previously reported coordinate activation of four of the five activities by the first substrate (3) is here extended by the observation of coordinate protection of all five activities by DAHP. The conformational change which was presumed in the earlier report to take place as a result of DAHP binding is clearly indicated by the marked changes in protease sensitivity.

The data are consistent with the "domain" theory of conjugate structure (5), as we have found that the order of sensitivities of the five activities remains the same with three different protease preparations, suggesting that similar regions of the conjugate are attacked. Gel electrophoresis studies are currently under way to determine if in fact the peptides of the arom conjugate formed by digestion with trypsin, chymotrypsin, and the N. crassa protease preparation are similar. These studies will also provide information concerning the nature of the protection by DAHP. Preliminary comparative sodium dodecyl sulfate and native gel electrophoretograms already suggest that DAHP not only protects from proteolysis per se but also protects by maintaining the integrity of the native structure once proteolysis has occurred.

Although it may be premature to argue that the protection exerted by DAHP is of physiological significance, the concentrations of DAHP required in vitro to protect are not unrealistic. Also, as mentioned earlier, we have found that lower concentrations of DAHP are effective in protecting against proteolytic inactivation when lower amounts of protease are used. This might be representative of the situation in vivo, where normally the arom conjugate would not be exposed to such high concentrations of protease. In addition, due to physical and metabolic compartmentation, it is possible that DAHP concentrations in the micro-environment could reach levels as high as 10 mM. Finally we consider it likely that the catalytic site and the site(s) responsible for activation (3) and/or coordinate protection are distinct. The K_m for catalysis is about 0.1 mM whereas the concentration of DAHP required for activation (3) and coordinate protection is more than tenfold higher than this value.

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