PHOSPHOCELLULOSE, AN AFFINITY CHROMATOGRAPHIC SYSTEM FOR CHORISMATE SYNTHASE AND THE AROMATIC COMPLEX OF NEUROSPORA CRASSA*

K. W. Cole and F. H. Gaertner

Biology Division, Oak Ridge National Laboratory, and The University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, Tennessee 37830

Received August 22,1975

SUMMARY: Unlike other enzymes of the aromatic multienzyme system, chorismate synthase and the aromatic complex of Neurospora crassa were found to bind to a column of cellulose phosphate and to elute at a relatively high concentration of phosphate (~0.2 M). The fact that other enzymes with similar ionic properties failed to bind to phosphocellulose suggests that the binding of the former two enzyme systems is due to a specific affinity for phosphate. This conclusion is not only supported by the fact that these same enzymes did not bind to a column of carboxymethyl cellulose, but also is consistent with the nature of the catalytic reactions of the enzymes. Both the shikimate kinase enzyme of the aromatic complex and chorismate synthase would be expected to have active sites which accomodate a phosphate moiety. We anticipate that other enzymes which involve phospho-substrates will also be amendable to this procedure.

INTRODUCTION

In previous studies on the aromatic multienzyme system of N. crassa, we have observed that 11 of 13 enzymes catalyzing the conversion of erythrose-4-phosphate and phosphoenolpyruvate to L-tryptophan bind to diethylaminoethyl (DEAE)-cellulose and elute in approximately the same concentration range (0.08–0.12 M) with a linear phosphate gradient (1, 2). In particular, the 5-enzyme aromatic complex and the 3-enzyme anthranilate synthase complex eluate nearly in coincidence (3).

Recently, a very active protease was demonstrated in N. crassa (4). The protease coincidently, has some of the same molecular properties as the aromatic enzymes. Fortunately, this protease is inactive during early stages of purification due to a highly efficient and specific natural inhibitor (4).

*Research sponsored by the Energy Research and Development Administration under contract with the Union Carbide Corporation.

In our own studies on this protease and its inhibitor (unpublished data), we have found that the protease does not bind to DEAE-cellulose and that the inhibitor binds only slightly (which, again fortuitously, protects the aromatic enzymes from proteolytic attack). However, some proteolytic activity may be unmasked at later stages in purification which could lead to artifactual results in studies concerning the structure and functions of the aromatic enzymes.

As a consequence of the above observations and conclusions, we have attempted to eliminate the protease from our preparations at an early purification state by taking advantage of the wide difference in the affinity of the aromatic enzymes and the protease in ion-exchange chromatography. Since the protease does not bind to DEAE-cellulose (an anion-exchange column), we anticipated that it would bind tightly to a cation-exchange column such as phosphocellulose. On the other hand, since the aromatic enzymes bind to DEAE-cellulose, we expected them to simply wash through the phosphocellulose, free of the protease.

Here we report the surprising result that two of the aromatic enzyme components, chorismate synthase and the aromatic complex, bind tightly to phosphocellulose. Our results indicate that this cation exchanger may serve as a affinity chromatographic system for these enzymes as well as for other similar types of enzymes.

MATERIALS AND METHODS

Growth of the Organism and Preparation of Extract: Wild-type N. crassa (strain 74A) was grown, harvested, lyophilized, extracted, treated with protamine sulfate, fractionated at 60% of saturation with ammonium sulfate, and desalted on a 4 x 90 cm column of Sephadex G-25 with 0.05 M potassium phosphate (pH 7), as described previously (1, 5). All buffers for extraction and chromatography contained 2×10^{-4} M dithiothreitol and 10^{-4} M ethylenediaminetetraacetic acid. All procedures were carried out at 0–5° C.

Phosphocellulose Chromatography: Cellulose phosphate (Whatman Column Chromedia P1 7.4 meq/g) was washed several times with deionized water, filtered on a Buchner funnel, resuspended in 0.25 M HCl, and allowed to stand for 30 min at 25° C. The cellulose

was then washed 6 times with deionized water, treated with 0.25 M NaOH, and equilibrated by repeatedly washing it with 0.01 M potassium phosphate (pH 6.5).

The desalted extract described above from 75 g of lyophilized mycelium was either chromatographed first on a 2 x 80 cm column of DEAE-cellulose (2) or was applied directly to a 2 x 80 cm column of phosphocellulose and eluted at 37 ml/hr with a 1500-ml linear gradient of 0.01–0.15 M potassium phosphate (pH 6.5). After a brief extension of the gradient with 0.15 M potassium phosphate, a second 1500-ml linear gradient of 0.15 M to 0.5 M potassium phosphate (pH 6.5) was used.

Carboxymethyl Cellulose Chromatography: Selectacel standard carboxymethyl cellulose (0.78 meq/g Schleicher and Schuell, Inc.) was washed with 0.05 M NaCl–0.25 M NaOH, filtered, washed 5 times with deionized water, and equilibrated to pH 6.5 with 0.01 M potassium phosphate buffer. A 2 x 80 cm column of CM cellulose was eluted with a linear gradient of 0.01–0.15 M potassium phosphate (pH 6.5), followed by a second gradient of 0.15–0.5 M potassium phosphate (pH 6.5). The desalted extract from 40 g of lyophilized mycelium was used.

Enzyme Assays: Anthranilate synthase complex (1), chorismate synthase (6), kynureninase (5), kynurenine formamidase (7), and aromatic complex (3) were assayed as described previously. The anthranilate synthase and dehydroshikimate reductase assays were used to identify the two complexes.

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*We have found it convenient to use a simple gradient extender between two successive linear gradients. It allows the column to operate without close supervision and eliminates the possibility that the column will run dry. In its simplest form, the extender is a 1-liter Erlenmeyer flask with a two-hole rubber stopper which secures two glass tubes (2 mm I.D.) approximately 30 cm in length. One tube contains a shutoff valve 10 cm from the stopper and is slightly longer (8–10 mm) than the second tube. When the system is used to extend a 0.01–0.15 M potassium phosphate gradient, the flask is filled with 0.15 M phosphate buffer, stoppered, and inverted into the 0.15 M container of the gradient-maker. The longest of the two tubes is held 3–5 mm off the bottom of the breaker. The distance between the ends of the two tubes determines the volume at which the extension system becomes operational. For example, a difference of 8–10 mm allows the volume in the 0.15 M container of the gradient-maker to lower to ~50 ml before additional buffer enters from the inverted Erlenmeyer flask.

RESULTS

In DEAE-cellulose chromatography, the anthranilate synthase complex, chorismate synthase, and the aromatic complex elute at approximately the same point in the linear phosphate gradient at 0.10–0.12 M phosphate (2). As can be seen in Fig. 1, on phosphocellulose the anthranilate synthase complex and aromatic complex are widely separated using the same phosphate gradient. Most unexpectedly, the aromatic complex and chorismate synthase bind tightly to phosphocellulose and elute exactly in coincidence at a concentration of approximately 0.2 M phosphate.

That this tight binding does not involve nonspecific ionic attractions may be seen by the facts that (i) in addition to anthranilate synthase, other enzymes with similar ionic properties

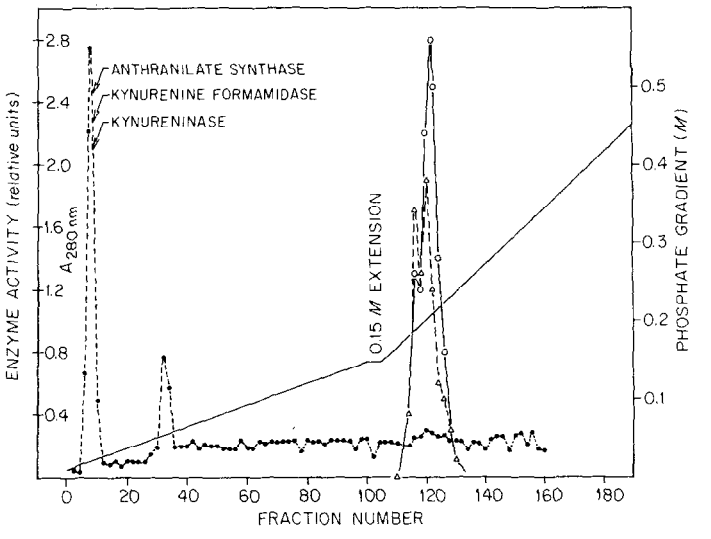

FIG. 1 Phosphocellulose chromatogram of an extract of N. crassa 74A prepared as described in "Methods". In this case the ammonium sulfate fraction was chromatographed first on DEAE-cellulose (see ref. 2, Fig. 3a), then fractions 90 through 140 were pooled, concentrated by ammonium sulfate precipitation at 60% of saturation, and desalted by gel filtration on a 2 x 80 cm column of G–25 prior to chromatography on DEAE-cellulose. In other experiments, with comparable results, the desalted ammonium sulfate fraction was placed directly on phosphocellulose without prior chromatography on DEAE-cellulose. The peak positions of kynureninase, kynurenine formamidase, and the anthranilate synthase complex are indicated by arrows. Chorismate synthase activity, O —— O; aromatic complex, △ —— △. Absorbance at 280 nm is indicated by broken line; phosphate gradient is given by solid line.

simply wash through the column, e.g., kynureninase and kynurenine formamidase and (ii) the majority of protein pooled from the 0.10–0.12 M region of the DEAE-cellulose phosphate gradient did not stick to phosphocellulose. Moreover, when carboxymethyl cellulose is used, the aromatic complex and chorismate synthase behave in the expected manner. They do not bind to this latter cation-exchange resin, but elute together with anthranilate synthase and other enzymes in the void volume (results not shown).

DISCUSSION

The fact that chorismate synthase and the aromatic complex bind to phosphocellulose, whereas other proteins with similar ionic properties do not, suggests that this cation exchanger is acting as an affinity chromatographic system for chorismate synthase and the aromatic complex. These enzymes potentially bind phosphate specifically by virtue of the fact that both have active sites which likely accommodate phosphate. See, for example, the shikimate kinase reaction (catalyzed by the aromatic complex) and the chorismate synthase reaction schemes depicted in Fig. 2. The reason these two enzymes elute from phosphocellulose at

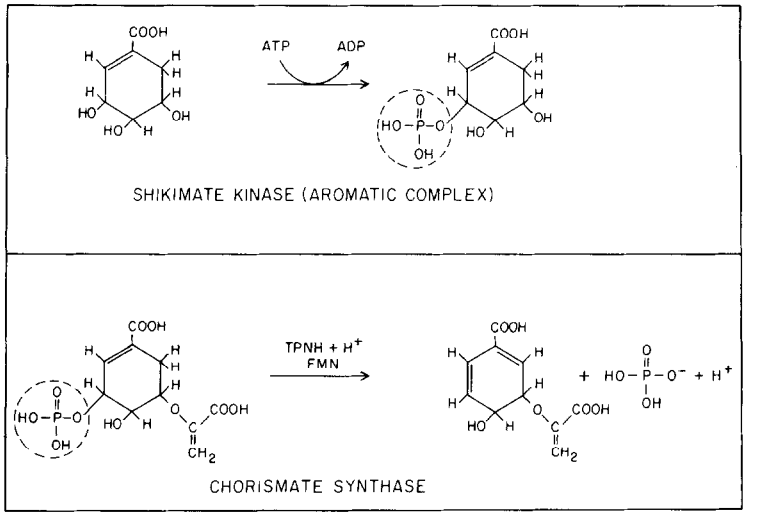

FIG. 2 Reactions catalyzed by the aromatic complex and chorismate synthase. Dashed lines emphasize the likelihood of a phosphate binding site on each enzyme.

exactly the same position in the phosphate gradient may be only coincidental, but the possibility of a specific interaction between the aromatic complex and the consecutive enzyme in the aromatic pathway, chorismate synthase, should not be overlooked.

We consider it likely that other enzymes of this type may bind in a similar fashion to phosphocellulose. In support of this notion, it has recently come to our attention that some phosphatases have a specific affinity for the cellulose phosphate matrix (8).

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