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ACTIVATION OF PREPHENATE DEHYDRATASE BY ADSORPTION TO AGAROSE DERIVATIVES[1]

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A series of potential affinity adsorbents for prephenate dehydratase were synthesized by coupling allosteric effectors to agarose with and without spacer arms. Only cationic ligands containing a hexamethylene spacer arm adsorbed the enzyme. Activity could be removed from aminohexyl- and glycyl-aminohexyl-agaroses with 0.5 M NaCl (with loss of activity), 0.4 M phosphate, or 140 mM hexanediamine, but was not removed from ala-aminohexyl-, phe-aminohexyl-, met-aminohexyl-, leu-aminohexyl-, or phenylpyruvyl-aminohexyl-agaroses or by Gly, Ala, Phe (inhibitor), Met or Leu (both activators). Urea (1.0 M), 1 mM phosphate, 50% glycerol in phosphate or prephenic acid (substrate) did not release enzymatic activity from any of the gels. The gel-bound enzyme was found to have enhanced activity comparable to that of the methionine-activated soluble enzyme. The site and mode of the enzyme immobilization are discussed in relation to the possible hydrophobic/ionic subunit–subunit interactions.

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Prephenate dehydratase (EC 4.2.1.51) from *Bacillus subtilis* is an allosteric enzyme in the pathway for phenylalanine biosynthesis (1). It catalyzes the conversion of prephenic acid to phenylpyruvate, and is regulated primarily by L-phenylalanine, a feedback inhibitor, and secondarily by the extra-pathway metabolites L-tryptophan, L-leucine, and L-methionine (1–5). The regulatory relationships among these amino acids form the basis for a balancing mechanism for hydrophobic amino acids referred to as "Metabolic Interlock" by Jensen (1).

In an attempt to purify the enzyme, which is available only in minute quantities from the source, a number of potential affinity adsorbents were prepared utilizing the known allosteric effectors. While a gel derivative applicable to purification of the enzyme was not realized, we did find that the adsorbed enzyme was activated regardless of the type of ligand employed.

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MATERIALS AND METHODS

Sepharose 4B was obtained from Pharmacia Fine Chemicals. All chemicals and solvents were reagent grade. Buffers were prepared with distilled deionized water.

Potassium prephenate was prepared and purified by a modification of the procedure of Cotton and Gibson (6, 7). Prephenate dehydratase was obtained from *B. subtilis*, strain NP-1 (5).

Preparation of Extracts

Cultures for cell-free extracts were grown in glucose-minimal salts medium (8) in 16-liter carboys at 37°C under forced aeration. Cells were harvested in the late exponential phase of growth by centrifugation, and washed at 4°C with an equal volume of 20 mM potassium phosphate, pH 7.5, containing 0.5 mM EDTA, 0.5 mM dithioerythritol, and 20% glycerol (standard buffer). The washed cells were diluted with the standard buffer to a concentration of 1 g/20 ml. Lysozyme (400 μg/ml) was added and the cell suspension was shaken at 37°C for 15 min. Deoxyribonuclease (10 μg/ml), ribonuclease (10 μg/ml), and $MgSO_4$ (10 mM) were then added to the final concentrations indicated, and incubated with shaking 10 min at 37°C. Cellular debris was removed by centrifugation at 47,000 *g* for 90 min at 4°C, and the supernatant was dialysed overnight against the standard buffer.

Assay for Prephenate Dehydratase

To 100 μl of the enzyme solution was added 100 μl of 160 mM methionine (in standard buffer). The mixture was incubated at 32°C for 20 min followed by the addition of 50 μl of 6.0 mM potassium prephenate (in standard buffer) to initiate the reaction. After 20 min, 1.75 ml of 2.5 N NaOH was added and the absorbance read at 320 nm. The concentration of the product, phenylpyruvate, was determined by referring to a standard curve.

Synthesis of Gel Derivatives

Agarose Activation. A slurry of Sepharose 4B (1 part packed gel/1 part water) was mixed with an equal volume of 0.5 N NaOH and reacted with epichlorohydrin (0.48 M) at 30°C for 4 h following the procedure of Nishikawa and Bailon (9). After washing, the appropriate amines were added as described below.

Amine Coupling. The washed epoxide-activated agarose was added to an equal volume of (a) 2 M NH_4OH (b) 2 M 1,6-hexanediamine in H_2O, (c) 0.05 M ε-aminocaproic acid in 0.25 M NaOH, or (d) 0.05 M L-amino acid in 0.25 M NaOH. The mixture was shaken at 30°C for 16 h, filtered and washed well with water, 2 M KCl, and water. *N*-succinyl-3-amino-2-hydroxypropyl-agarose was prepared by reacting 3-amino-2-hydroxypropyl-agarose with succinic anhydride according to the method of Cuatrecasas (10). Aminoalkyl agaroses were acetylated with a 750-fold excess of acetic anhydride in 0.5 N NaOH at 4°C.

Ligand Attachment. Carboxyl-containing ligands were coupled to the amino-agaroses via *N*-hydroxysuccinimide esters (10). The α-amino groups of L-amino acids were blocked with o-nitrophenylsulfenylchloride (11). After coupling, the blocking group was quantitatively removed by treatment with sodium thiosulfate (12). L-Amino acids were also coupled through the amino group to the *N*-hydroxysuccinimide ester of *N*-succinyl-3-amino-2-hydroxypropyl-agarose (13).

Structures of the various gel derivatives are shown in Table 1.

Ligand Analysis

Free amino groups were qualitatively detected with 2,4-6-trinitrobenzenesulfonate by the method of Inman and Dintzis (14). Actual concentrations of amino or carboxyl groups were determined by potentiometric titration in 0.2 M KCl. Amino acid concentrations were determined, when possible, by standard amino acid analysis (Beckman Model 120C Amino Acid Analyzer) after hydrolysis of the gel *in vacuo* in 6 N HCl at 110°C for 20 h.

Affinity Testing of Agarose Derivatives

The gel (0.25 ml) was preequilibrated with standard buffer and mixed with 1.0 ml of crude enzyme extract in 12 × 75-mm test tubes. The tubes were mixed for 1 h at 25°C on a Burrell "Wrist action" shaker. The gel beads were settled by centrifugation, and an aliquot of the supernatant was assayed for enzymatic activity. Unsubstituted agarose, which does not bind the enzyme, was used as a control. Enzymatic activity in the supernatant of the control is referred to as 100%.

After the incubation period, the supernatant was siphoned off and the gel–enzyme complex was washed with 12–15 volumes of standard buffer. Eluting conditions were tested in the same manner, and were done at 25°C for 30 min unless otherwise noted.

TABLE 1. **Screening Results of Potential Affinity Adsorbents for Prephenate Dehydratase**

	Percent unbound relative activity[a]
Group 1 (G = Agarose—$OCH_2CH(OH)CH_2$—)	
G—NH_3^+	93
G—$NHCOCH(NH_3^+)CH_2CH(CH_3)_2$	90
G—$NHCOCH(NH_3^+)CH_2Ø$	90
Group 2 (G = Agarose—O—$CH_2CH(OH)CH_2$—)	
G—$NH_2^+(CH_2)_5CO_2^-$	107
G—$NH_2^+CH(CO_2^-)CH_3$	78
G—$NH_2^+CH(CO_2^-)CH_2CH(CH_3)_2$	108
G—$NH_2^+CH(CO_2^-)CH_2$—(3-indolyl)	96
G—$NH_2^+CH(CO_2^-)CH_2CH_2SCH_3$	102
G—$NH_2^+CH(CO_2^-)CH_2Ø$	104
Group 3 (S = —$HNCOCH_2CH_2CO$—)	
G—S—$NHCH(CO_2^-)CH_3$	93
G—S—$NHCH_2CO_2^-$	92
G—S—$NHCH(CO_2^-)CH_2CH(CH_3)_2$	95
G—S—$NHCH(CO_2^-)CH_2CH_2SCH_3$	93
G—S—$NHCH(CO_2^-)CH_2Ø$	98
Group 4[b] (H = —$NH_2^+(CH_2)_6$—)	
G—H—NH_3^+	1.6
G—H—$NHCOCH_2NH_3^+$	3.6
Group 5[b]	
G—H—$NHCOCH(NH_3^+)CH_3$	8.1
G—H—$NHCOCH(NH_3^+)CH_2CH(CH_3)_2$	1.6
G—H—$NHCOCH(NH_3^+)CH_2CH_2SCH_3$	15
G—H—$NHCOCH(NH_3^+)CH_2Ø$	1.6
Group 6[b]	
G—H—$NHCOCOCH_2Ø$	21
Group 7[b]	
G—$N(COCH_3)(CH_2)_6NHCOCH_3$	90

[a] Each gel derivative was screened for enzyme binding ability as described under Materials and Methods. Percent unbound activity refers to relative enzymatic activity remaining in the supernatant after incubation and centrifugation, normalizing the activity of the control (unsubstituted agarose) to 100%.

[b] In the text and in subsequent tables, these gels will be referred to by the following abbreviations: For group 4 gels, GH—NH_3^+, GH-gly; group 5, GH-ala, GH-leu, GH-met, GH-phe; group 6, GH-ØPA; group 7, diacetyl-GH.

Gel enzymatic activity was measured by adding 0.90 ml of standard buffer and 0.10 ml of 6.0 mM prephenate to the washed gel-enzyme complex. The mixture was shaken at 25°C for 1 h, then centrifuged. A 0.20 ml aliquot of the supernatant was added to 1.80 ml of 2.5N NaOH and the absorbance read at 320 nm. The activity in the supernatant of the control (unsubstituted agarose with crude enzyme extract and no activators) was normalized to a value of 1.0.

RESULTS

Affinity Testing of Adsorbents

All the potential adsorbents were initially assayed for binding ability by an equlibrium (static) method. Table 1 summarizes the relative affinity of prephenate dehydratase for each gel derivative. Gel derivatives which are positively charged but lack a hexamethylene spacer arm (group 1), zwitterionic (group 2), or are negatively charged (group 3) did not bind the enzyme. Positively charged gels containing the hexamethylene spacer (groups 4 and 5) adsorbed the enzyme, whereas the acetylated aminohexyl derivative (gel 7) did not. Thus, the initial screening of the various gel derivatives demonstrated that the proper adsorbent must contain the hexamethylene spacer and be cationic. All subsequent studies were done with this set of adsorbents, which is comprised of gels coupled to enzyme modifiers (GH-leu, GH-met, and GH-phe), the enzymatic reaction product (GH-ØPA), and to control compounds (GH-NH_3^+, GH-gly, and GH-ala). (See Table 1 footnote for use of abbreviations.)

Assay of Gel-Bound Activity

At the concentrations of protein employed in this study, the enzymatic activity was barely observable in the absence of an activator. Methionine was therefore routinely added to the incubation mixture for the assay of soluble prephenate dehydratase. Gel-bound enzymatic activity was assayed by incubating the gel–enzyme complex with substrate and measuring the product concentration in the supernatant. As the control, the enzyme extract was treated with unsubstituted agarose, and its activity was defined as 1.0. The second control was identical except that methionine was added to the incubation mixture. As shown in Table 2 the binding of the enzyme to all the gels except GH-ØPA resulted in an activation on the same order as that realized by the addition of activator to the soluble enzyme. Assay of the supernatant showed that the enzyme was not released from the gel by this treatment. The activation was also observed when the enzyme was bound to

TABLE 2. **Enzymatic Activity of Prephenate Dehydratase Immobilized onto Group 4, 5, and 6 Gel Derivatives**

Ligand	Ligand concentration (μeq/ml)	Relative activity[a]
Unsubstituted	—	1.0
Unsubstituted and Met	—	76
GH—NH_3^+	16	81
GH-gly	16	81
GH-ala	13	70
GH-leu	12	74
GH-phe	10	50
GH-met	7	47
GH-ØPA	16	4.3

[a] Gel bound enzymatic activity was determined as described under Materials and Methods. Activity of the control (unsubstituted agarose) was normalized to 1.0.

the gel in the presence of inhibitors or activators. When phenylalanine was present in the assay medium, however, no activity was detected. The addition of methionine to the assay solution did not effect further activation. The lower level of activation observed on GH-phe and GH-met may be the result of a lower degree of ligand substitution.

Enzyme purified by conventional methods (15) was bound and activated to the same degree as crude enzyme, ruling out the possibility that the effect was caused by other protein components in the extract.

Elution Attempts

The gel derivatives in the aminohexyl series were grouped according to their behavior with various eluting conditions, summarized in Table 3. The group 4 derivatives were intended to be controls. Although they adsorbed the same quantity of enzyme, their behavior with the various elution solvents was quite different as compared to the group 5 gels. Under equilibrium conditions, enzymatic activity was released from group 4 gels by 0.14 M hexanediamine or 0.4 M phosphate: 0.5 M NaCl also released the enzyme, but with loss of activity (NaCl has been shown to inhibit the activity of soluble prephenate dehydratase) (16). Neither these solvents nor solutions of the appropriate counterligand (or other hydrophobic amino acids,

TABLE 3. **Effect of Solvents on the Elution and Activity of Immobilized Prephenate Dehydratase**

Treatment[a]	Gel derivative group		
	4	5	6
0.6 mM prephenic acid	NR/A	NR/A	NR
0.5 M NaCl	R[b]	NR/LA	NR
0.4 M phosphate	R	NR/LA	
140 mM 1,6-hexanediamine	R	NR/LA[c]	
Counterligand[d]	NR/A	NR/LA	
Other hydrophobic amino acids[d]	NR/A	NR/LA[e]	
1.0 mM phosphate	NR/A	NR/A	NR
50% glycerol	NR/A	NR/A	NR
60% glycerol + counterligand	NR/A	NR/A	
1.0 M urea	NR/A	NR/A	NR

[a] Each treatment proceeded for 30 min as described under Materials and Methods in standard phosphate buffer, except where phosphate or glycerol concentrations were altered.
[b] R: released from gel; NR/A: not released from gel/gel activity unchanged; NR/LA: not released from gel/gel activity decreased.
[c] Except GH-phe which equals NR/A. Approximately 15% of the activity was released into the supernatant, and relative gel-bound activity was 85%.
[d] [Phe] = 10 mM, [Leu], [Met], [Gly], [ØPA] = 50 mM.
[e] With methionine treatment, gel activity decreased only 20–30%; treatment with phenylalanine or leucine resulted in a 50–70% decrease in gel activity.

nor the substrate) released the enzyme from the group 5 set of derivatives. In addition, treatment of the group 5 gel–enzyme complex under these conditions resulted in a decrease in gel-bound activity of 55–90% with salts, 70–90% with hexanediamine, 50–70% with Phe or Leu, and 20–30% with Met. Elution of the enzyme from any of the gels could not be induced by a decrease in buffer ionic strength or by the addition of glycerol or 1 M urea to the standard buffer, but there was no decrease in gel-bound enzymatic activity. (In solution, the addition of 1 M urea caused a 50% decrease in activity (16)).

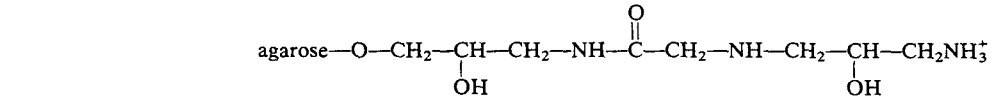
DISCUSSION

It is logical to design an affinity chromatographic purification for an enzyme utilizing supports that contain inhibitors, activators, or substrates for the enzyme. Hopefully one can develop a system in which the enzyme is biospecifically adsorbed and desorbed in a process that is uncomplicated by nonspecific ionic or hydrophobic interactions. In an attempt to detect an

appropriate affinity chromatographic system for prephenate dehydratase, a series of synthetic adsorbents were screened for binding ability.

The equilibrium (static) method of affinity testing employed in this study has proved very useful for the rapid screening of a large number of potential adsorbents. In this manner it was possible to quickly determine the structural requirements necessary to bind prephenate dehydratase. It is apparent that the only gel derivatives that adsorbed the enzyme were amphipathic; that is, composed of a hydrophobic hexamethylene spacer arm and a cationic terminal functionality. Those derivatives which also included a hydrophobic amino acid seemed to bind the enzyme even tighter, for the 0.4 M phosphate buffer did not elute the enzyme from these gels under the testing conditions. Although most of the eluting buffers chosen did not desorb the enzyme (Table 3), their effect on gel-bound enzymatic activity did lend some insight as to the nature of the interaction between enzyme and adsorbent. In those cases where the enzyme was eluted (i.e., from the aminohexyl-agarose by high phosphate concentration), the purification factor was similar to that gained by conventional chromatography on DEAE-cellulose (9 to 13-fold) (15). Although a useful purification method did not evolve, an unusual phenomenon was observed: immobilized prephenate dehydratase had as much as 80 times more activity than the soluble enzyme in the absence of effectors. The investigation, therefore, turned toward identifying the nature of this unusual effect.

It is probable that the principal interaction between the gels in groups 4 and 5 and the enzyme is ionic/low affinity hydrophobic. The acetylated derivative (diacetyl-GH) did not bind the enzyme, nor did the cationic hydrophilic derivative (17) shown below (16):



Such synergism between ionic and hydrophobic binding effects has been noted previously by Hofstee (18, 19), Yon (20), Nishikawa (21, 22), Porath (23) and others (24, 25). The major difference between group 4 and group 5 gels may be the greater contribution of the hydrophobic interactions in the latter. While high phosphate concentration released the enzyme from group 4 gels, it merely deactivated the gel-bound enzyme on group 5 derivatives. At higher salt concentrations, the protein may re-equilibrate to a predominantly hydrophobic binding situation with subsequent denaturation at the protein–hydrocarbon interface.

When the gel-enzyme complexes were incubated under hydrophobic solvent conditions, such as decreasing salt concentration, increasing glycerol concentrations, or the addition of urea to the buffer, the enzyme was still not

eluted from the gel but it was also not deactivated. Apparently, a re-equilibration to tighter hydrophobic bonding is discouraged. An apparent inhomogeneity of binding sites was observed by Hofstee with bovine serum albumin adsorbed onto 4-phenyl-*n*-butylamine-substituted agarose (18). Part of the protein was eluted with a 0–1.0 M NaCl gradient. The addition of ethylene glycol (to 50%, v/v) to the buffer was required to release the remaining protein. This phenomenon was interpreted as an interchange from an electrostatic interaction at low ionic strength to a hydrophobic interaction at high ionic strength.

Inasmuch as the gel-bound enzyme is catalytically active and was not eluted by substrate, it is certain that the binding did not occur via the active site or an inhibitor site. This is supported by the fact that the adsorbed enzyme was also activated. One exception was observed with GH-ØPA, which exhibited very little gel-bound and no supernatant activity. Phenylpyruvate, the reaction product, has also been shown to be an inhibitor of prephenate dehydratase (5). It is possible that the enzyme was bound to this ligand at the active site or an inhibitor site, preventing catalysis, or it may be nonspecifically bound at hydrophobic sites and deactivated or denatured. The likelihood that the enzyme bound to group 4 and 5 gels at an activator site cannot be ruled out. The most potent activators for the soluble enzyme are amino acids bearing alkyl side chains, such as L-leucine, L-norleucine, L-methionine, L-ethionine and 2-amino-DL-caprylic acid(5). Two other structural requirements for activation are the presence of an α-hydrogen and the free α-amino group. The resemblance of these alkyl amino acids to the hexamethyleneamine spacer arm is noteworthy. The degree of activation observed for the bound enzyme was the same as methionine-activated soluble enzyme. Methionine, however, did not protect the enzyme from adsorption, and none of the hydrophobic amino acids at concentrations tested were able to release the gel-bound protein. This could be explained by the existence of multiple binding sites with different equilibrium constants. Activity was eluted from group 4 gels with 1,6-diaminohexane, but it is not known whether this was a specific or ionic interaction. Phosphate buffer at an equivalent ionic strength eluted the enzyme. In solution, it has been shown that 1,6-diaminohexane is not an effector of prephenate dehydratase (16).

It is known that amphiphiles bind to a number of proteins accompanied in some cases by drastic conformational changes (26). Serum albumin, a relatively hydrophobic protein, is a common example. The basis for such associations is thought to be nonspecific hydrophobic interactions augmented by specific polar (ionic) attachments (26). Many enzymes can be immobilized "irreversibly" to alkylamino-coupled agarose or nonionic hydrophobic derivatives of insoluble supports and still retain catalytic

activity (18,23,27–29). Hofstee (18) has speculated that such enzymes may have "nonfunctional" binding sites which are involved in associations with intracellular matrices. It is conceivable that hydrophobic forces, with or without ionic support, are involved in the association of protein subunits (18,26). It was reported by Pierson and Jensen (5) and confirmed by Riepl and Glover (15) that methionine, an activator of prephenate dehydratase, also enhances the oligomerization of the enzyme to a higher molecular weight form. If the immobilized hydrophobic/ionic ligand were to bind to subunit oligomerizing sites, one might speculate that conformational changes leading to enzyme activation might be induced. Work is in progress to establish the type of relationship between the association and activation processes. It would be of interest to examine the immobilization of other oligomerizing enzymes on these gel derivatives and screen for possible rate enhancements.

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