Protein Chromatography on Adsorbents with Hydrophobic and Ionic Groups

SOME PROPERTIES OF *N*-(3-CARBOXYPROPYONYL)AMINODECYL-SEPHAROSE AND ITS INTERACTION WITH WHEAT-GERM ASPARTATE TRANSCARBAMOYLASE

By ROBERT J. YON and ROGER J. SIMMONDS
*School of Biological Sciences, Thames Polytechnic, Wellington Street,
London SE18 6PF, U.K.*

(Received 23 June 1975)

1. The charge state of two derivatives of Sepharose prepared by the CNBr activation method were studied by acid–base titration and by ion-exchange chromatography. Dodecyl-Sepharose exhibited cationic groups ($21\ \mu\text{mol/ml}$ of settled gel; $pK_a = 9.6$) that were tentatively assigned to the coupling isourea group. 2. CPAD-Sepharose [*N*-(3-carboxypropionyl)aminodecyl-Sepharose] has anionic (carboxyl) groups ($pK_a = 4.5$) and cationic groups ($pK_a = 9.6$) in roughly equal concentrations ($13\text{--}15\ \mu\text{mol/ml}$ of settled gel); the cationic group was again tentatively assigned to the coupling group. CPAD-Sepharose is slightly negatively charged at pH 7.0 and substantially negatively charged at pH 8.5. 3. The pK_a values of dodecyl-Sepharose and CPAD-Sepharose are unaffected by a 100-fold increase in the concentration of KCl. 4. CPAD-Sepharose has considerable affinity for wheat-germ aspartate transcarbamoylase at pH 8.5 when the adsorbent and enzyme are both negatively charged. The interaction involves the C_{10} chain but is relatively moderate compared with C_{10} chains associated only with positive charge. 5. Desorption of the enzyme adsorbed to CPAD-Sepharose can be achieved by raising the pH to increase the electrostatic repulsion, or by introducing the detergent sodium deoxycholate. Acetone and butan-1-ol also weaken the adsorption at pH 8.5. 6. High concentrations of sodium acetate or sodium phosphate induced the enzyme to bind more tightly to CPAD-Sepharose. 7. These results are discussed in terms of a ‘repulsion-controlled’ model of hydrophobic chromatography.

The purification of water-soluble proteins by ‘hydrophobic chromatography’ has attracted the attentions of several groups of workers and a considerable literature on the subject now exists (see references 1–10 in Er-el & Shaltiel, 1974; also Hjertén, 1973; Jennissen *et al.*, 1973; Yon, 1974; Grover & Hammes, 1974; Hofstee, 1974; Hjertén *et al.*, 1974; Williams, 1974; Henderson *et al.*, 1974; Raibaud *et al.*, 1975; Schöpp *et al.*, 1975; Jennissen & Heilmeyer, 1974, 1975). A few reports have described the use of hydrophobic adsorbents that were free of charged groups (Hjertén *et al.*, 1974; Wilchek, 1974). However, most adsorbents in use have been derived by the CNBr procedure (Axen *et al.*, 1967) which is known to introduce positively charged coupling groups into the adsorbent matrix (Hofstee, 1973a; Jost *et al.*, 1974). Additional positive or negative charges have been introduced to produce a variety of adsorbents able to interact non-specifically with proteins by a combination of hydrophobic and electrostatic forces. Depending on pH and the isoelectric points of individual proteins, hydrophobic interactions may be reinforced by electrostatic attractive forces (Hofstee, 1973a; Shaltiel & Er-el, 1973; Jost *et al.*, 1974) or opposed

by electrostatic repulsive forces (Yon, 1972a, 1974; Rimerman & Hatfield, 1973). Provided that binding takes place under the latter conditions, it is arguable that the binding forces are predominantly hydrophobic and may be controlled by varying the repulsion between protein and adsorbent, by varying the pH, for example. The present paper is a report of further studies on a system believed to be representative of this type of protein chromatography.

Preliminary studies using bovine serum albumin and the adsorbent CPAD-Sepharose* were interpreted as indicating that for this system, at any rate, hydrophobic bonding could occur in the presence of limited electrostatic repulsion between the protein ($pI = 4.9$) and the carboxyl groups on the adsorbent (Yon, 1972a). However, these studies did not consider the possibility of positive charges on the adsorbent; with more recent reports on the introduction of such charged groups by the CNBr coupling method (see references quoted earlier) it therefore became necessary to take a closer look at the charge state of CPAD-Sepharose and its involvement in the chromatographic process. The present paper is a

* Abbreviation: CPAD-Sepharose, *N*-(3-carboxypropionyl)aminodecyl-Sepharose.

report of some of these studies in which wheat-germ aspartate transcarbamoylase was used as the test protein. The use of an enzyme enables judgements to be made on the activity state of the protein after exposure to the hydrophobic adsorbent, a feature missing from earlier work with serum albumin. In addition this enzyme is stable at room temperature and alkaline pH (up to about 10) (Yon, 1973), a condition likely to favour the development of negative charge on the enzyme and the adsorbent. Other studies on this enzyme and its chromatography on hydrophobic adsorbents have been described elsewhere (Yon, 1974).

Experimental

Materials

Sephacrose 4B was obtained from Pharmacia (G.B.) Ltd., Ealing, London W5, U.K. 1,10-Diaminodecane and 1,2,4,5-benzenetetracarboxylic acid were obtained from Ralph Emmanuel, Alperton, Middx., U.K. Succinic anhydride, aminomethane, 1,2-diaminoethane, 1-aminodecane, L-aspartic acid, sodium deoxycholate, $(\text{NH}_4)_2\text{SO}_4$, KCl, organic solvents and all buffer components were obtained from BDH, Poole, Dorset, U.K. CNBr was obtained from Koch-Light, Colnbrook, Bucks., U.K. Carbamoyl phosphate was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. DEAE-cellulose and CM-cellulose were from Whatman Biochemicals, Maidstone, Kent, U.K.

Buffers

Tris buffers were made up to the stated concentration in Tris base and then adjusted to the stated pH with acetic acid (Tris-acetate buffer) or HCl (Tris-HCl buffer). Most chromatographic experiments were performed in a mixture referred to as standard buffer containing 0.1M-Tris base and 0.1M-glycine; it was adjusted to the stated pH with acetic acid or 10M-NaOH. Sodium phosphate buffers were made up to the stated concentration in NaH_2PO_4 and adjusted with 10M-NaOH.

Formation of Sepharose derivatives

Sephacrose 4B was activated with CNBr (15g/100ml of settled gel) and coupled with 1,10-diaminodecane or 1-aminodecane at saturating concentration in aqueous solution containing 20% (v/v) ethanol. The methods of Cuatrecasas (1970) were followed. Sepharose coupled to 1-aminodecane is referred to as dodecyl-Sepharose, and Sepharose coupled to 1,10-diaminodecane is referred to as 10-aminodecyl-Sepharose (Fig. 1). At the saturating concentrations used in coupling, 1,10-diaminodecane is coupled essentially through one amino group (see the Results section). CPAD-Sepharose was prepared from washed 10-aminodecyl-Sepharose by treatment with solid succinic anhydride (0.2g/ml of settled gel), the mixture being stirred for 3–4h at room temperature while the pH was kept steady at pH6.0 by the dropwise addition of 10M-NaOH. All the gels were given extensive washes (with at least 50 times the gel

Fig. 1. Structures of some derivatives of Sepharose 4B with hydrophobic groups

The polysaccharide matrix is represented by hatching. The principle charged coupling group is taken to be the *N*-substituted isourea following Wilchek (1974). The charge state is shown as at pH7.0.

volume) in (1) distilled water, (2) 0.2M-trisodium phosphate containing 5% (v/v) butan-1-ol and (3) distilled water, in that order. Dodecyl-Sepharose was additionally washed in 96% (v/v) ethanol. The gels were stored as suspensions in distilled water adjusted to pH 5.0 and containing 0.02% (w/v) sodium azide as an antimicrobial agent. For storage over long periods (several weeks or months) these suspensions were frozen at -20°C . References to the 'settled gel' allude to gels that have been allowed to settle undisturbed for at least 16 h (i.e. overnight).

Preparation of crude extract of aspartate transcarbamoylase from wheat germ

A partially purified preparation of aspartate transcarbamoylase from wheat germ was obtained as described previously (Yon, 1972b). The enzyme was purified to the acetone-fractionation stage. Less than 1% of the protein in this extract consists of the enzyme.

Acid-base titration of Sepharose derivatives

The gels were titrated in the presence of various concentrations of KCl. Before titration, they were washed in large volumes of KCl solution at the appropriate concentration, over a Büchner funnel and then suspended in the same solution. A Vibrette model 46A pH-meter (EIL Instruments, Richmond, Surrey, U.K.) was fitted with a glass electrode and used to measure pH to 0.01 unit. The instrument was calibrated with commercial pH standards. The titration vessel was magnetically stirred and thermostatically controlled at $25 \pm 0.1^{\circ}\text{C}$. Approx. 25 ml (settled vol.) of each gel was suspended in the appropriate KCl solution to a total volume of 50.0 ml. The pH was adjusted cautiously to just below 3, and was then titrated to more alkaline values by the addition of small volumes of NaOH solution, by using a Hamilton microsyringe. The pH was raised in 0.2 unit increments from pH 3 to 11. After each addition of NaOH, 1 min was allowed for the pH to equilibrate. A blank titration, using 50.0 ml of the KCl solution alone, was also carried out. For each pH interval of 0.2 unit, the difference between the test and blank titration gave the number of groups that were deprotonated within that interval. The results are presented in differential form (Fig. 2) which has the advantage that the distribution of titratable groups over the pH range is readily seen. As the difference error rapidly increases on approaching the extremes of the pH range, only data between pH 3.4 and pH 10.6 are presented. An indication of the repeatability of our results is given in the titration of CPAD-Sepharose (Fig. 2c) where the vertical bars at selected pH values show the range of four determinations of which the mean is plotted.

Column chromatography of small organic/inorganic ions

To test the charge state of Sepharose derivatives their ion-exchange behaviour towards small ions was examined. Proteins or other large ions could not be used, as ion-exchange effects were likely to be obscured by hydrophobic interactions. Low-ionic-strength buffers ($I = 0.001\text{--}0.002\text{ mol/l}$) were used. The experiments were performed at room temperature on columns of 8.0–8.5 ml bed volume and 0.8 cm diameter, that were equilibrated with 0.001 M-Tris-acetate buffer or 0.001 M-sodium phosphate buffer. Samples were introduced in 0.2 ml of the same buffer with the pH readjusted if necessary. If the small-ion sample failed to appear in the first 4–6 column volumes of effluent, it was eluted with 10 mM-NaCl in buffer. The flow rate was 1.3 ml/min and fractions of 1.3 ml were collected. The samples that were used, and the methods of assay, were as follows. Benzenetetracarboxylate ($0.4\text{ }\mu\text{mol}$) was monitored as E_{280} . Aminomethane and 1,2-diaminoethane ($2\text{ }\mu\text{mol}$ each) were assayed as described by Fields (1971). In $(\text{NH}_4)_2\text{SO}_4$ ($2\text{ }\mu\text{mol}$) NH_4^+ ions were assayed by Nessler's reagent and SO_4^{2-} ions were precipitated with an excess of BaCl_2 and the turbidity of the suspension (as apparent absorption at 400 nm) was compared with a calibration curve. Cl^- ions in NaCl ($2\text{ }\mu\text{mol}$) were similarly determined by precipitation with AgNO_3 .

Chromatography of the crude enzyme extract

Details are given with each experiment (Figs. 4–6). In general small columns were used. The flow rate was approx. 1 ml/min. Enzyme activity in the column effluent fractions was determined as described previously (Yon, 1972b) except that the use of HClO_4 was omitted. Protein was monitored continuously as E_{280} ; occasionally it was determined by the method of Lowry *et al.* (1951) with bovine serum albumin that had been heated to constant weight at 60°C as the standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide gels (7.5%) were made up as described in the Shandon Instruction booklet supplied with the Shandon apparatus (Shandon Scientific Co., London N.W.10, U.K.) except that the gel and tank buffers were carefully adjusted to pH 8.5 before use. Samples of protein ($50\text{--}100\text{ }\mu\text{g}$) were applied in 5% sucrose. The gels were stained with Coomassie Blue and destained by the method of Weber & Osborn (1969). Aspartate transcarbamoylase bands were shown by incubating the gels at room temperature for 30 min in a solution containing 1.0 mM-L-aspartate, 1.0 mM-carbamoyl phosphate, 2 mM- CaCl_2 and 0.1 M-glycine, the whole adjusted to

pH 10 with NaOH. The reaction product, phosphate, is deposited as a white band of calcium phosphate.

Results

Titration profiles of Sepharose derivatives

Titration profiles of dodecyl-Sepharose and CPAD-Sepharose are compared with unmodified Sepharose in Fig. 2. These titrations were performed in 0.15M-KCl. Unmodified Sepharose has a negligible number of groups that dissociate between pH 3.4 and 10.6. Dodecyl-Sepharose and CPAD-Sepharose both showed a major titration peak corresponding to a pK_a of 9.5. The concentration of groups dissociating between 7.6 and 10.6 (in $\mu\text{mol}/\text{ml}$ of settled gel) was 21 ± 2 for dodecyl-Sepharose and 15 ± 2 for CPAD-Sepharose. The carboxyl groups of CPAD-Sepharose are evident as the titration peak corresponding to a pK_a of approx. 4.5. The concentration of groups titrating between pH 3.4 and 6.6 (assumed to be all carboxyl groups) was $13 \pm 2 \mu\text{mol}/\text{ml}$ of settled gel. Repeated re-treatment of CPAD-Sepharose with succinic anhydride (see the Experimental section) did not significantly decrease the size of the alkaline peak for this gel nor increase the size of the acid peak, showing that within the limits of detection by titration, all the free amino groups of 10-aminododecyl-Sepharose were succinylated in the formation of CPAD-Sepharose. Also, the approximate equality of the alkaline and acid peaks suggests that, in the coupling of 1,10-diaminododecane to the activated Sepharose, the major portion of the

diamine is coupled through one amino group only, since the cationic group is probably introduced into the gel at this stage (see the Discussion section).

Dodecyl-Sepharose and CPAD-Sepharose were also titrated in the presence of 0.01M-, 0.10M- and 1.0M-KCl. The effects of the different salt concentrations on the pK_a values of the main groups in these gels (estimated as the pH value at the centre of the titration peak) are tabulated in Table 1. Portions of CM-cellulose and DEAE-cellulose containing approximately the same numbers of titratable groups as the sample of CPAD-Sepharose were titrated under identical conditions. These results are also included in Table 1. The theory of ion-exchange resins (Kunin, 1958a,b) predicts that the pK_a values (pH at which a group is 50% protonated) of immobilized groups involved in ion exchange should be sensitive to increasing concentrations of exchangeable ions in the solvent phase, which should depress the pK_a of an anionic group and elevate that of a cationic group. For univalent solvent ions and weakly acidic or basic exchanger groups, the displacement is approx. 1 pK_a unit for a 10-fold change in ion concentration. Table 1 shows that our technique could demonstrate this effect quite clearly for CM-cellulose (0.6 pK_a unit decrease per 10-fold increase in concentration of KCl) and for DEAE-cellulose (0.8 pK_a unit increase for the same change in concentration of KCl). However, no significant displacement trends could be detected in the pK_a values of dodecyl-Sepharose or CPAD-Sepharose.

Ion-exchange behaviour of CPAD-Sepharose and dodecyl-Sepharose

Experiments were conducted to examine possible ion-exchange behaviour in these gels towards small inorganic and organic ions that lacked extensive hydrophobic moieties (and whose interactions with the adsorbents would not, therefore, be obscured by hydrophobic interactions). At low buffer concentrations ($I \leq 0.002 \text{ mol/l}$) and low sample loading, ion-exchange effects were observable. No ion-exchange

Fig. 2. Differential titration of (a) Sepharose 4B, (b) dodecyl-Sepharose and (c) CPAD-Sepharose in the pH range 3.4–10.6

For details see the text.

Table 1. Effects of increasing KCl concentration on the dissociation constants (pK_a) of titratable groups in various adsorbents

For further details see the text.

	pK_a values (± 0.2 unit) in:		
	0.01M-KCl	0.10M-KCl	1.0M-KCl
Dodecyl-Sepharose	9.7	9.6	9.7
CPAD-Sepharose	4.5	4.6	4.8
CM-cellulose	5.0	4.4	3.8
DEAE-cellulose	8.4	9.2	10.0

Fig. 3. Chromatography of small ions on CPAD-Sepharose at pH 8.5

Chromatography of the following small ions is shown: (a) 1,2,4,5-benzenetetracarboxylate, (b) Cl^- , (c) $(\text{NH}_4)_2\text{SO}_4$ (NH_4^+ ion trailing), (d) aminomethane, (e) 1,2-diaminoethane. See the Experimental section for further details.

effects were observed at $I = 0.020$ mol/l or greater. When the sample loading conditions were increased above $4 \mu\text{mol}$ of sample ion per ml of column volume (only univalent sample ions were tested) a significant fraction of the exchangeable ion appeared in the void volume and was therefore not retarded by the column. It is noteworthy that this loading limit is considerably smaller than the number of potential exchanger groups in both dodecyl-Sepharose and CPAD-Sepharose, judged by the titration results.

The ion-exchange behaviour of several small ions under the low-ionic-strength low-loading conditions described above, were examined. Dodecyl-Sepharose behaved towards these ions as a typical anion-exchanger at pH 7.0, suggesting that the groups responsible for the major titration peak in Fig. 2(b) are cationic, and probably nitrogenous. It is assumed that the alkaline peak of CPAD-Sepharose is due to similar cationic groups. At pH 7.0, CPAD-Sepharose showed both anion- and cation-exchange characteristics, all the small ions tested (benzenetetracarboxylate, aminomethane, diaminoethane, NH_4^+ and

SO_4^{2-}) being retarded to varying degrees on a column of this adsorbent. On raising the pH to 8.5, however, CPAD-Sepharose behaved as a typical cation-exchanger. This property of CPAD-Sepharose is illustrated in Fig. 3. The change in behaviour between pH 7.0 and 8.5 is due to the deprotonation of a significant fraction (about $4 \mu\text{mol}/\text{ml}$ of settled gel) of the groups responsible for the alkaline peak in Fig. 2(c).

Charge state of wheat-germ aspartate transcarbamoylase at pH 8.5

The enzyme was purified as described by Yon (1974) and subjected to polyacrylamide-gel electrophoresis with the gel and tank buffers adjusted to pH 8.5. It migrated towards the anode and is therefore negatively charged at this pH. In Tris-acetate buffer (0.1 M) at pH 8.5, it is weakly adsorbed from a crude extract by DEAE-cellulose but not by CM-cellulose, confirming its negative charge at this pH.

Adsorption of the enzyme

The abilities of dodecyl-Sepharose, CPAD-Sepharose and 10-aminododecyl-Sepharose to adsorb the enzyme from the crude extract were compared at pH 8.5 and room temperature. Dodecyl-Sepharose bound the enzyme extremely tightly; all the enzyme activity from 5 column volumes of the crude extract was adsorbed, and none of it could be desorbed by irrigating with 10 column volumes of buffered 1 M-NaCl, by saturating the column with deoxycholate, by raising the pH to 10.0 or by lowering it to 6.0. It is probable that the enzyme is extensively denatured by interaction with dodecyl-Sepharose. Similar behaviour had been noted previously with decyl-Sepharose (1-aminodecane coupled to CNBr-activated Sepharose) (Yon, 1972c). The enzyme was also relatively strongly bound by 10-aminododecyl-Sepharose; again, all the activity from 5 column volumes of the crude extract was adsorbed. About 10% of the adsorbed activity was desorbed by irrigation with buffered 0.5 M-NaCl, but attempts to elute more activity by using 1 M-NaCl, by saturating the column with deoxycholate or by raising or lowering the pH were unsuccessful. CPAD-Sepharose bound the enzyme with considerable strength but with decreased capacity compared with dodecyl-Sepharose and 10-aminododecyl-Sepharose. The enzyme activity from 1.5–2 column volumes of the crude extract was the most that this adsorbent would bind. By using desorption methods described in the next section, recoveries from this material were high; up to 100% could be recovered from small trial columns, but larger preparative columns gave recoveries of 50–60%.

Desorption of the enzyme bound to CPAD-Sepharose

After adsorption of the enzyme at pH 8.5, it was found possible to desorb it in several different ways. The enzyme could always be desorbed by raising the pH. When this was done in a continuous pH gradient, the enzyme was desorbed at pH 9–9.6 (see, for example, Fig. 6a). Significantly, this is the pH range in which the major cationic group in the adsorbent (see titration results) is discharged. The bound enzyme was not desorbed on lowering the pH to 5.5, the lower limit of stability of the enzyme activity.

When the enzyme was introduced on to the column in the presence of 0.2% (w/v) sodium deoxycholate, it was prevented from binding (Fig. 4b). The presence of butan-1-ol (7%, v/v) or of acetone (10%, v/v) when the enzyme was introduced weakened its binding to the column (Figs. 4c and 4d). When the enzyme was allowed to bind in the absence of any of these compounds, and the compounds then introduced into the eluent in an attempt to desorb the enzyme, it was desorbed very effectively by deoxycholate (0.2%, w/v), the enzyme emerging in a sharp peak, but was desorbed very slowly by butan-1-ol (7%, v/v) or acetone (10%, v/v), the enzyme emerging in a very broad shallow peak.

There is a considerable degree of specificity in the ability of deoxycholate to desorb the enzyme. A number of other compounds with hydrophobic moieties, including some selected for their resemblance to the binding groups on CPAD-Sepharose, were unable to desorb the bound enzyme when used at the same concentration (5 mM) as deoxycholate. The compounds tested were palmitate and 11-amino-undecanoate (as sodium salts), 1-aminododecane, 1,10-diaminododecane and 1,12-diaminododecane. The neutral detergents Triton X-100, Brij 35 and polyethylene glycol, used at much higher concentrations (5%, w/v or v/v) were likewise unable to desorb the bound enzyme or to prevent the enzyme from binding. Sodium dodecyl sulphate (0.2%, w/v) was able to desorb the enzyme but also extensively denatured it.

Ionic-strength effects

The effects of sodium acetate and sodium phosphate on the binding of the enzyme to CPAD-Sepharose were studied in detail. NaCl inhibited the enzyme at moderate concentrations and its effects are therefore not reported here.

Fig. 5 shows that when loaded with 0.5 column volume of the crude enzyme extract at pH 8.5 and room temperature, CPAD-Sepharose was able to bind 100% of the sample enzyme activity in the presence of very high ionic strengths of sodium acetate and sodium phosphate, showing that non-specific ionic forces (i.e. between oppositely charged

groups) play an insignificant role in the binding of the enzyme. Examination of the protein profiles of Fig. 5 shows that a high proportion (about 40%) of the total protein in the crude extract was similarly able to resist desorption by high-ionic-strength solutions. All of the bound enzyme, but not all of the bound protein, could be recovered on desorption by low-ionic-strength buffer at pH 10.5.

Fig. 4. Effects of sodium deoxycholate, butan-1-ol and acetone on the binding of the enzyme to CPAD-Sepharose

Columns of CPAD-Sepharose (bed volume 3.0 ml, diameter 0.8 cm) were equilibrated with (a) standard buffer, pH 8.5, (b) 0.2% (w/v) sodium deoxycholate in standard buffer, pH 8.5, (c) 7% (v/v) butan-1-ol in standard buffer, and (d) 10% (v/v) acetone in standard buffer, pH 8.5. Samples of the crude enzyme preparation were diluted twofold into buffer with appropriate additions to give the same final concentration as in the equilibrating solution, and 3.0 ml of the sample mixture was loaded on to each column. The columns were then eluted with 12 column volumes of equilibrating solution, and finally with standard buffer adjusted to pH 10.5. Fractions of volume 3 ml were collected. The temperature was 22°C. Enzyme activity (●) is shown.

Fig. 5. Effects of (a) sodium phosphate and (b) sodium acetate on the chromatography of the crude enzyme extract on CPAD-Sepharose

Columns of CPAD-Sepharose (bed volume 1.5 ml, diameter 0.8 cm) were equilibrated with various concentrations of sodium phosphate or sodium acetate in standard buffer diluted fourfold and adjusted to pH 8.5. Each column was loaded with 0.75 ml of the crude enzyme extract that had previously been adjusted to pH 8.5 and the appropriate concentration of salt. The column was then washed with 4 column volumes of the equilibrating solution. Protein and enzyme activity that failed to appear in the effluent up to this point were defined as 'bound'. Finally the columns were washed with standard buffer at pH 10.5 to displace the enzyme. Open symbols refer to protein (E_{280}) and closed symbols to enzyme activity. The graphs show (○, ●) percentage of protein and activity adsorbed at pH 8.5 and (△, ▲) percentage of protein and activity recovered at pH 10.5. The temperature was 22°C.

Fig. 6. Increased affinity of the enzyme for CPAD-Sepharose induced at high ionic strength

Columns of CPAD-Sepharose (bed volume 2.0 ml, diameter 0.8 cm) were equilibrated with (a) standard buffer, pH 8.5, (b) 2.0 M-sodium acetate in standard buffer adjusted to pH 8.5 and (c) 0.7 M- NaH_2PO_4 in standard buffer adjusted to pH 8.5. Each column was loaded with 6.0 ml of the crude enzyme that had previously been adjusted to pH 8.5 and the appropriate concentration of salt. After a brief wash with the equilibrating buffer the columns were developed with increasing pH gradients up to pH 10.5 (----) in which salts were included as appropriate. Finally the columns were eluted with standard buffer at pH 10.5; in (b) and (c) the added salt was omitted from this wash at the point shown by the arrow. Protein (E_{280} ; ○) and enzyme activity (●) are shown. Fractions of volume 3 ml were collected. The temperature was 22°C.

Experiments were performed to find out whether the enzyme bound in the presence of 0.7 M-sodium phosphate (near saturation for this salt at pH 8.5) or 2.0 M-sodium acetate was more or less tightly bound than at low ionic strength. The results are presented in Fig. 6. Columns were deliberately overloaded so that the amount of enzyme activity bound could be found as the difference between that in the sample and that in the unbound void-volume peak. The effects of phosphate and acetate were compared with a control. At high ionic strength there was a substantial increase in the amount of enzyme adsorbed compared with the control. Not only was more enzyme bound, but the enzyme was bound more tightly at high ionic

strength. This is deduced from the observation that, whereas pH values lower than 10.5 resulted in desorption of all of the bound enzyme at low ionic strength (Fig. 6a), none of the enzyme was desorbed by pH 10.5 at high ionic strength (Figs. 6b and 6c). As soon as the ionic strength of the eluent was decreased, however (indicated by the arrow in Figs. 6b and 6c) there was an immediate desorption of nearly all the bound enzyme. A substantial amount of protein in the crude extract behaved in a manner similar to the enzyme in these experiments. A considerable separation of the enzyme from contaminating proteins was achieved by developing columns, to which crude enzyme had been adsorbed at high ionic strength, with decreasing ionic-strength gradients.

Discussion

Charge state of dodecyl-Sepharose and CPAD-Sepharose

Studies on the charge properties of monoaminoalkanes attached to Sepharose by the CNBr method have been reported by Hofstee (1973*a,b*, 1974), Wilchek (1974) and Jost *et al.* (1974). Our results with dodecyl-Sepharose are in qualitative agreement with these reports and suggest, as they do, that this type of adsorbent is positively charged at near-neutral pH. Our pK_a value of 9.6 is somewhat lower than the value of 10.5 for butyl-Sepharose reported by Jost *et al.* (1974). There have been suggestions that the main cationic group concerned is the *N*-substituted isourea coupling derivative (Axen & Ernback, 1971; Guilford, 1973; Wilchek, 1974; Jost *et al.*, 1974) as indicated in Fig. 1.

The carboxyl groups of CPAD-Sepharose, introduced by the treatment with succinic anhydride, are clearly associated with the pK_a value of approx. 4.5 in Fig. 2(c). The pK_a value of 9.6 for this gel we assign to the coupling isourea group by analogy with dodecyl-Sepharose and other monoaminoalkyl derivatives of Sepharose. The distribution of titratable groups in Fig. 2(c) suggests that CPAD-Sepharose has approximately equal numbers of anionic and cationic groups, and this is qualitatively supported by our observation that at pH 7.0 the gel shows weak ion-exchange ability towards both small cations and small anions. However, preliminary experiments using the technique of particle electrophoresis (R. J. Simmonds, unpublished work) have indicated that at pH 7.0 there is a small excess of negative charge on the gel particles. On raising the pH from 7.0 to 8.5, approx. $4\mu\text{mol}$ of cationic groups per ml of settled gel are deprotonated, which should place a substantial negative charge on the gel particles. This expectation is qualitatively supported by the weak ion-exchange chromatography of small ions (Fig. 3) in which it is seen that the column behaves as a typical cation-exchanger at pH 8.5, and by the preliminary particle-electrophoresis experiments mentioned above. All our present evidence, therefore, indicates that this gel is negatively charged under the conditions (pH 8.5 and above) used in studies of its interactions with the enzyme.

An effect about which we can only speculate at the present time is the failure of dodecyl-Sepharose and CPAD-Sepharose to show the ionic-strength-dependent displacement of pK_a values (Table 1) that one would expect from potential ion-exchangers (Kunin, 1958*a,b*). One possible explanation is that only a minority of the titratable groups on these gels are able to exchange K^+ and Cl^- ions (used in our titration experiments) with the bulk solvent. This interpretation would be consistent with our finding that under very-low-ionic-strength conditions, the

columns are overloaded at a sample loading (about $4\mu\text{mol}$ of univalent ion per ml of gel volume) that is considerably lower than one might expect from the number of potential exchanger groups ($21\mu\text{mol/ml}$ for dodecyl-Sepharose and $13\text{--}15\mu\text{mol/ml}$ for CPAD-Sepharose) indicated by titration. The ions used in these experiments were all as large or larger than the K^+ and Cl^- ions. It would be of interest to examine the effects of ion size and of the large hydrophobic groups on the gel, in an attempt to resolve this anomaly.

Interaction of CPAD-Sepharose with wheat-germ aspartate transcarbamoylase

With a few exceptions, the enzyme-adsorbent interactions were studied in the pH range 8.5–10.5. Evidence discussed above shows that the adsorbent is negatively charged at pH 8.5 and hence at all higher pH values. Similarly, electrophoretic and ion-exchange experiments showed the enzyme to be negatively charged over the same pH range. We suggest that the resulting electrostatic repulsion modifies what would otherwise be an extremely strong and destructive hydrophobic interaction. We describe hydrophobic chromatography under these conditions as being 'repulsion-controlled'.

When proteins interact non-specifically with alkyl-Sepharoses in which the positive coupling group is the only charged group, hydrophobic and electrostatic attractive forces co-operate in protein-binding, the hydrophobic contribution increasing with the length of the alkyl chain (Hofstee, 1974). The interaction is often strong enough to extensively denature proteins, particularly at the longer chain lengths (C_8 or greater). These reports are supported and extended by our experiences with decyl-Sepharose (Yon, 1972*c*) and dodecyl-Sepharose (the present paper). These adsorbents have high binding capacities for proteins and no active aspartate transcarbamoylase could be recovered from them, confirming that C_{10} and C_{12} alkyl-Sepharoses interact extremely strongly with this and other proteins, probably denaturing them by a 'detergent-like' (Wilchek, 1974) effect. However, when the distal ends of these alkyl chains terminate in ionic groups, and particularly in negatively charged groups, profound changes in their behaviour ensue. A comparison of the properties of 10-aminodecyl-Sepharose and of CPAD-Sepharose is instructive. These derivatives both have C_{10} chains, but at pH 8.5 CPAD-Sepharose is negatively charged whereas 10-aminodecyl-Sepharose is positively charged. At this pH the binding capacity of CPAD-Sepharose for the enzyme is less than half of that of 10-aminodecyl-Sepharose, but recovers from CPAD-Sepharose are high (100% in favourable cases) whereas they are low (10% or less) from 10-aminodecyl-Sepharose. The lower binding capacity and milder interaction

with the enzyme that characterize CPAD-Sepharose we ascribe to the charge-repulsion effect. The greater binding capacity of 10-aminodecyl-Sepharose is probably due to the co-operation of hydrophobic and ion-exchange effects, but is obtained at the cost of a much higher loss of enzyme activity through denaturation.

The effects of high ionic strength on the interaction of the enzyme and CPAD-Sepharose are noteworthy. Several reports have appeared describing salt-induced binding of proteins to hydrophobic adsorbents. Nicolas *et al.* (1972) showed that $(\text{NH}_4)_2\text{SO}_4$ induced the binding of oestradiol-17 β dehydrogenase to oestrone-Sepharose. Rimerman & Hatfield (1973) showed phosphate-induced binding of bacterial proteins to valyl-Sepharose; their adsorbent, like CPAD-Sepharose, was probably negatively charged at the operating pH. Hjertén *et al.* (1974) using hydrophobic adsorbents that were electrically uncharged, reported increased protein adsorption in the presence of high concentrations of NaCl. Raibaud *et al.* (1975) induced the binding of proteins to positively charged alkyl-Sepharoses by the use of high concentrations of $(\text{NH}_4)_2\text{SO}_4$. The general effect shown by these and the present studies is that a variety of inorganic salts, at sufficiently high concentrations, will induce hydrophobic interactions between proteins and hydrophobic adsorbents; the available data suggest that the effect is independent of the nature of the inorganic salt or of the charge state of the adsorbent. These effects are not unexpected, since less-polar interactions between proteins and adsorbents, like the 'salting-out' of proteins from solution, are favoured by the decrease in solvating water available to them in the presence of high concentrations of very soluble inorganic ions. In addition to this general effect, however, another factor could operate under repulsion-controlled conditions such as exist during our experiments using CPAD-Sepharose, when high salt concentrations can lead to tighter hydrophobic binding by shielding repellent charges from each other, as first suggested by Rimerman & Hatfield (1973).

Hofstee (1974) has presented evidence that alkyl-Sepharoses are heterogeneous in binding sites for a pure homogeneous protein. It is reasonable to suppose that CPAD-Sepharose will similarly be heterogeneous in sites capable of binding aspartate transcarbamoylase. In the micro-environments of these binding sites one would expect heterogeneity to arise from steric considerations as well as from variations in the relative contributions of hydrophobic bonding, electrostatic attractive forces including ion-exchange and ion-dipolar interactions (Porath & Fryklund, 1970) and electrostatic repulsion forces. A number of lines of evidence suggest that above pH 8.5 in the present case, electrostatic attractive forces play an insignificant role, and that

the main binding forces are hydrophobic, limited to varying degrees by electrostatic repulsion. These lines of evidence are (1) the weakening of enzyme binding by the inclusion of low-polarity compounds (acetone and butan-1-ol) and of the detergent, sodium deoxycholate, (2) the fact that high salt concentrations increase rather than decrease the enzyme-adsorbent affinity and (3) the profound change in behaviour of the hydrophobic C_{10} chain when the negatively charged *N*-(3-carboxypropionyl)amino group is introduced.

Moderation of the potentially extremely strong hydrophobic bonding by long-chain alkyl groups is the most useful characteristic of adsorbents represented by CPAD-Sepharose. They also permit the use of a variety of methods for selective desorption of bound proteins, including increasing pH, decreasing salt concentration and mild detergents. The general usefulness of CPAD-Sepharose and its C_6 , C_8 and C_{12} homologues in enzyme purification has been demonstrated in this laboratory, where several enzymes and other proteins (including wheat-germ aspartate transcarbamoylase) have been purified or are approaching purity by methods based on the use of these adsorbents (R. J. Yon, R. J. Simmonds, J. E. Grayson & C. F. Potter, unpublished work).

We are grateful for the financial support of the S.R.C. (Enzyme Chemistry and Technology Committee).

References

- Axen, R. & Ernback, S. (1971) *Eur. J. Biochem.* **18**, 351–360
- Axen, R., Porath, J. & Ernback, S. (1967) *Nature (London)* **214**, 1302–1304
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065
- Er-el, Z. & Shaltiel, S. (1974) *FEBS Lett.* **40**, 142–145
- Fields, R. (1971) *Biochem. J.* **124**, 581–590
- Grover, A. K. & Hammes, G. G. (1974) *Biochim. Biophys. Acta* **356**, 309–318
- Guilford, H. (1973) *Chem. Soc. Rev.* **2**, 249–270
- Henderson, G. B., Shaltiel, S. & Snell, E. E. (1974) *Biochemistry* **13**, 4335–4338
- Hjertén, S. (1973) *J. Chromatogr.* **87**, 325–331
- Hjertén, S., Rosengren, J. & Pahlman, S. (1974) *J. Chromatogr.* **101**, 281–288
- Hofstee, B. H. J. (1973a) *Biochem. Biophys. Res. Commun.* **50**, 751–757
- Hofstee, B. H. J. (1973b) *Anal. Biochem.* **52**, 430–448
- Hofstee, B. H. J. (1974) in *Immobilised Biochemicals and Affinity Chromatography* (Dunlap, R. B., ed.), pp. 43–59, Plenum Press, New York
- Jennissen, H. P. & Heilmeyer, L. M. G. (1974) *FEBS Lett.* **42**, 77–80
- Jennissen, H. P. & Heilmeyer, L. M. G. (1975) *Biochemistry* **14**, 754–760
- Jennissen, H. P., Horl, W. H. & Heilmeyer, L. M. G. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 236–237
- Jost, R., Miron, T. & Wilchek, M. (1974) *Biochim. Biophys. Acta* **362**, 75–82

- Kunin, R. (1958a) *Ion Exchange Resins*, 2nd edn., pp. 34–39, John Wiley and Sons, New York
- Kunin, R. (1958b) *Ion Exchange Resins*, 2nd edn., pp. 57–59, John Wiley and Sons, New York
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Nicolas, J. C., Pons, M., Descomps, B. & Crastes de Paulet, A. (1972) *FEBS Lett.* **23**, 175–179
- Porath, J. & Fryklund, L. (1970) *Nature (London)* **226**, 1169–1170
- Raibaud, O., Hogberg-Raibaud, A. & Goldberg, M. E. (1975) *FEBS Lett.* **50**, 130–134
- Rimmerman, R. A. & Hatfield, G. W. (1973) *Science* **182**, 1268–1270
- Schöpp, W., Meinert, S., Thyfronitou, J. & Aurich, H. (1975) *J. Chromatogr.* **104**, 99–104
- Shaltiel, S. & Er-el, Z. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 778–781
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wilchek, M. (1974) in *Immobilised Biochemicals and Affinity Chromatography* (Dunlap, R. B., ed.), pp. 15–31, Plenum Press, New York
- Williams, K. W. (1974) *Lab. Pract.* **23**, 521–524
- Yon, R. J. (1972a) *Biochem. J.* **126**, 765–767
- Yon, R. J. (1972b) *Biochem. J.* **128**, 311–320
- Yon, R. J. (1972c) Ph.D. Thesis, University of London
- Yon, R. J. (1973) *Biochem. J.* **131**, 699–706
- Yon, R. J. (1974) *Biochem. J.* **137**, 127–130