Lerman, L. S. (1953), *Proc. Nat. Acad. Sci. U. S. 39,* 232.
Lowry, O. H., Rosebrough, M. J., Farr, A. L., and Randall, R. S. (1951), *J. Biol. Chem. 193,* 265.
McCormick, D. B. (1965), *Anal. Biochem. 13,* 194.
Porath, J., Axen, R., and Ernback, S. (1967), *Nature (London) 215,* 1491.
Shaltiel, S., and Er-el, Z. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 778.
Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966), *Biochemistry 5,* 2108.
Shaltiel, S., and Patchornik, A. (1963), *J. Amer. Chem. Soc. 85,* 2799.
Snell, E. E., and Di Mari, S. J. (1970), *Enzymes, 3rd Ed., 2,* 335.
Wilchek, M., and Gorecki, M. (1969), *Eur. J. Biochem. 11,* 491.

ω-Aminoalkylagaroses in the Purification of L-Histidinol-phosphate Aminotransferase†

Gary B. Henderson,‡ Shmuel Shaltiel,§ and Esmond E. Snell*

ABSTRACT: A homologous series of ω-aminoalkylagaroses was tested in the purification of L-histidinol-phosphate aminotransferase (EC 2.6.1.9) from *Salmonella typhimurium.* Out of this series, the hexyl derivative was found to be the most suitable for this purpose, providing a purification of 15-fold in one step. This purification factor is very similar to that obtained by affinity chromatography. Combining the biospecific step with chromatography on ω-aminohexylagarose results in an enzyme preparation which is homogeneous when subjected to gel electrophoresis and has a specific activity of 40 units/mg, compared with 53 units/mg for the crystalline enzyme.

Using classical methods of enzyme purification (Henderson and Snell, 1973), L-histidinol-phosphate aminotransferase (EC 2.6.1.9) from *Salmonella typhimurium* was recently purified to homogeneity. In the preceding paper (Shaltiel *et al.,* 1974) we have described a biospecific method for the purification of this enzyme, using L-histidinol phosphate coated agarose. By this procedure, a 15.5-fold purification was achieved in one step, but the enzyme had a specific activity of only 15 units/mg, compared with 53 units/mg reported for the crystalline enzyme (Henderson and Snell, 1973).

In view of the remarkable purifications achieved recently with homologous series of alkyl- or ω-aminoalkylagaroses (Er-el *et al.,* 1972; Shaltiel *et al.,* 1973; Shaltiel, 1974a,b), we attempted to apply these new columns to the purification of L-histidinol-phosphate aminotransferase and thus compare their effectiveness with that of affinity chromatography.

Materials and Methods

Preparation of crude extracts from *Salmonella typhimurium,* assay of L-histidinol-phosphate aminotransferase, determination of protein concentrations, and gel electrophoresis were carried out by the methods quoted in the preceding paper (Shaltiel *et al.,* 1974). ω-Aminoalkylagaroses were prepared as described previously (Shaltiel and Er-el, 1973) except for the following modifications: the α,ω-diaminoalkane was dissolved in each case in a solvent composed of equal volumes of *N,N*-dimethylformamide and 0.1 M $NaHCO_3$ (pH 9.0) and the pH of the solution was readjusted to that value, if necessary. If a precipitate formed (with the higher members in the series) the suspension was warmed to 60° and then cooled rapidly before mixing with the activated agarose. After coupling at 22° and washing, each column was equilibrated with the buffer chosen for chromatography. DEAE-cellulose (DE 52) was purchased from Whatman.

Results

In order to establish which member within the homologous series Seph-C_n-NH_2[1] is most suitable for the purification of L-histidinol-phosphate aminotransferase, we used an exploratory kit (Shaltiel, 1974a,b) containing eight different small columns of ω-aminoalkylagaroses. These agarose derivatives differed in the number of carbon atoms (n) in their hydrocarbon side chains. Aliquots of the crude extract (0.1 ml) were applied on each of these columns. The amount of excluded aminotransferase activity was measured and plotted as per cent activity *vs. n.* As seen in Figure 1A, Seph-C_6-NH_2 retained all the aminotransferase activity when the columns were run with buffer A described in the legend to the figure. However, if the same series of columns were run with a buffer which was identical with A but contained also 0.25 M NaCl (Figure 1B), part of the aminotransferase activity was excluded by Seph-C_6-NH_2, indicating that increasing the ionic strength decreases retention of the enzyme and thus promotes elution. Under the conditions where the enzyme is retained by Seph-C_6-NH_2, ~75%

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received August 14, 1973.* Supported in part by Grants AM 01448 and AI 01575 from the U. S. Public Health Service.

‡ Present address: Scripps Clinic and Research Foundation, La Jolla, Calif.

§ Permanent address: The Weizmann Institute of Science, Rehovot, Israel.

[1] Abbreviations used are: Seph-C_n-NH_2, Sepharose 4B activated with CNBr and reacted with an α,ω-diaminoalkane n-carbon atoms long.

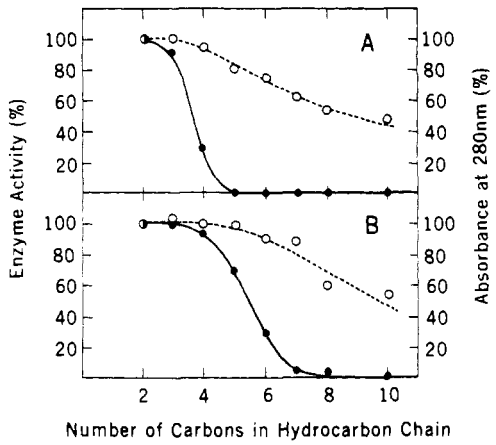

FIGURE 1: Selecting an ω-aminoalkylagarose for chromatography of L-histidinol-phosphate aminotransferase. A kit of Seph-C_n-NH_2 columns (n = 0-10) was equilibrated at 22° with (A) a buffer composed of Na_2HPO_4 (50 mM) and 2-mercaptoethanol (10 mM) adjusted to pH 7.0 with citric acid, (B) the above buffer with NaCl (0.25 M) added. Aliquots (0.1 ml) of the crude extract (dialyzed against the appropriate buffer in each case) were applied on each of the columns (4 × 0.5 cm); the first 1.6 ml was collected and the protein concentration (- -O) as well as aminotransferase activity (-●) were determined.

of the total protein is excluded by the column, as indicated by the absorbance at 280 nm (Figure 1A).

This result prompted us to attempt purification of L-histidinol-phosphate aminotransferase by applying the crude extract on the column at low ionic strength and then eluting the enzyme by gradually increasing the ionic strength of the buffer. Indeed a considerable purification could be achieved (Figure 2A). The crude extract obtained from *Salmonella typhimurium* had a specific activity of 0.33 unit/mg. Dialyzing it against a sodium acetate buffer (pH 5.0) (Henderson and Snell, 1973) raised the specific activity to 0.97 unit/mg. Subsequently chromatography on Seph-C_6-NH_2 resulted in an enzyme preparation with a specific activity of

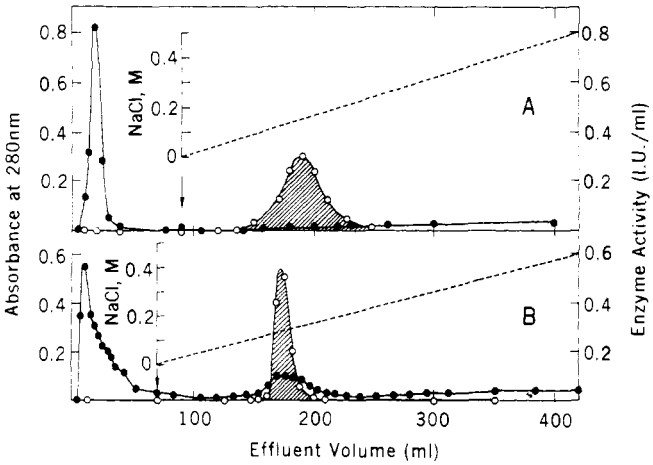

FIGURE 2: Chromatography of the crude extract on (A) Seph-C_6-NH_2 and (B) DEAE-cellulose. A sample of the crude extract (3 ml containing 17 mg of protein) was applied on each of the above mentioned columns which were identical in size (9 × 1.8 cm). The columns were equilibrated at 22° with a buffer composed of Na_2HPO_4 (50 mM) and 2-mercaptoethanol (10 mM) adjusted to pH 7.0 with citric acid. Unadsorbed protein was washed off and then (arrow) a linear NaCl gradient (up to 0.5 M, in the same buffer) was applied (200 ml in each reservoir). Fractions of (A) 5.0 ml or (B) 3.5 ml were collected, and their absorbances at 280 nm (●) as well as their aminotransferase activity (O) were monitored. The flow rates were 2.5 ml/min for A and 1.75 ml/min for B.

TABLE I: Comparison of Chromatography Procedures in the Purification of L-Histidinol-phosphate Aminotransferase.[a]

Column	Sp. Act.[b] (units/mg)	Purification Factor[c]	Yield of Enzyme[d] (%)
Seph-C_6-NH_2	14.8	15	99
DEAE-cellulose	7.5	7.5	98
L-Histidinol phosphate coated agarose[e]	15.0	15.5	125[f]
L-Histidinol phosphate coated agarose, then Seph-C_6-NH_2	40.0	45	94

[a] The extracts used in these experiments were dialyzed against a pH 5 buffer before purification by the indicated procedure. [b] Crystalline L-histidinol-phosphate aminotransferase preparations were reported to have specific activities up to 53 units/mg (Henderson and Snell, 1973). [c] During the chromatography steps. [d] Based on activity measurements. [e] Results from Shaltiel *et al.* (1974). [f] This measurement reflects inaccuracies in determining specific activities in crude preparations, which may contain inhibitory components.

14.8 units/mg. This represents an ~15-fold purification in the chromatography step and an ~45-fold overall purification.

In view of the fact that this enzyme had previously been purified about sixfold using DEAE-cellulose (Henderson and Snell, 1973) it was interesting to compare the efficiency of the Seph-C_6-NH_2 column with an identical-sized column of DEAE-cellulose under the same conditions. As seen in Figure 2B, DEAE-cellulose does indeed afford purification, but the enzyme fractions (shaded area) seem to have a lower specific activity (note the difference in the ratio between enzyme activity and absorbance at 280 nm). On a quantitative basis, Seph-C_6-NH_2 yielded an enzyme preparation with a specific activity twice as high as that obtained from DEAE-cellulose (Table I). Analysis by gel electrophoresis shows (Figure 3) that the enzyme obtained from DEAE-cellulose (gel C) contains protein impurity bands which are absent in the Seph-C_6-NH_2 preparation (gel B).

In the preceding paper (Shaltiel *et al.*, 1974) we have described an affinity chromatography procedure for the purification of L-histidinol-phosphate aminotransferase which resulted in an enzyme preparation with a specific activity of only 15 units/mg (Table I) compared with 53 units/mg reported for the crystalline enzyme (Henderson and Snell, 1973). We therefore attempted to further purify the enzyme obtained from the affinity chromatography column by using Seph-C_6-NH_2 or DEAE-cellulose. As seen in Figure 4, further purification was achieved in both cases. However, while the DEAE-cellulose increased the specific activity of the enzyme up to 22.6 units/mg, the Seph-C_6-NH_2 column raised the specific activity up to 40 units/mg and the resulting enzyme was homogeneous as judged by gel electrophoresis (Figure 3, gel D).

Discussion

The development of biospecific methods for the chromatography of proteins (Cuatrecasas and Anfinsen, 1972) has considerably reduced the number of steps required for protein purification. This approach (affinity chromatography)

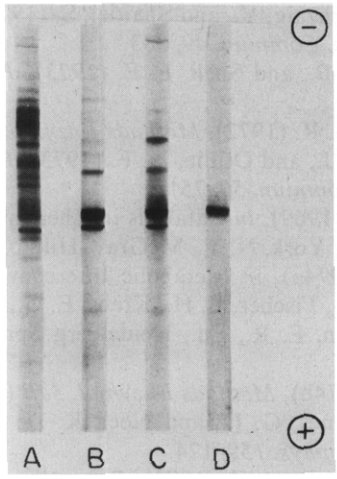

FIGURE 3: Purification of L-histidinol-phosphate aminotransferase by various chromatographic procedures as monitored by gel electrophoresis. Gel A contained 85 μg of crude extract; gel B contained 22 μg of enzyme preparation purified on Seph-C_6-NH_2; gel C contained 19 μg of enzyme preparation purified by passage on DEAE-cellulose; gel D contained 5.6 μg of enzyme which was first purified by passage through L-histidinol phosphate coated agarose and then through Seph-C_6-NH_2.

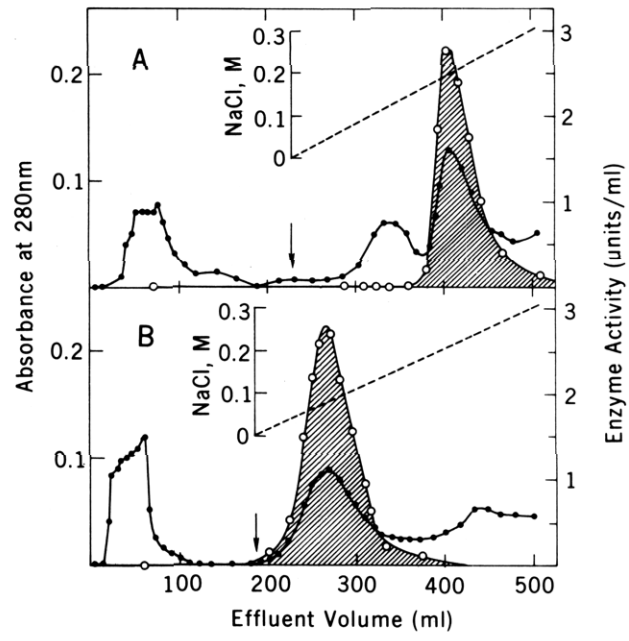

FIGURE 4: Comparison of DEAE-cellulose (A) and Seph-C_6-NH_2 (B) in their ability to further purify L-histidinol-phosphate aminotransferase obtained by affinity chromatography. Pooled fractions (total volume 98 ml; protein concentration 0.32 mg/ml) obtained from L-histidinol phosphate coated agarose (Shaltiel *et al.*, 1974; Figure 3B) were applied on the two columns mentioned above (49 ml on each). The columns (9 × 1.8 cm) were equilibrated (22°) with a buffer composed of Na_2HPO_4 (25 mM) and 2-mercaptoethanol (10 mM) adjusted to pH 7.0 with citric acid. Unadsorbed protein was washed off and then (arrow) a linear NaCl gradient (up to 0.3 M, in the same buffer) was applied. Fractions of (A) 6.0 ml or (B) 5.0 ml were collected and their absorbance at 280 nm (●) as well as their aminotransferase activity (○) were monitored.

is now widely used, due to the introduction of simple and efficient procedures for the activation of agarose (Axen *et al.*, 1967). Nevertheless, one still has to synthesize a specific column for the purification of each protein. Also, this technique is not always applicable because of the lability of some ligands and because of the fact that many proteins bind their substrates with rather high dissociation constants.

In contrast, alkyl- or ω-aminoalkylagaroses of the type Seph-C_n-X (Er-el *et al.*, 1972; Shaltiel and Er-el, 1973) can be used for the purification of a large variety of proteins. This technique can be tailored for each individual case by (a) choosing the homologous series to be used (the best *X*), (b) selecting the most effective member in the series (the best *n*), and (c) adjusting the loading and eluting conditions (ionic strength, buffer composition, pH, organic solvents, and temperature). These selections can be made rapidly and with small amounts of crude extracts by using an exploratory kit of columns (Shaltiel, 1974a,b).

The columns used in the present work (Seph-C_n-NH_2), having amino groups at the tip of their hydrocarbon "arms," may derive their discrimination power from ionic as well as hydrophobic interactions. It could be argued, for example, that the columns with longer "arms" are more effective since the amino groups at the tip of these "arms" can reach out farther and interact with less accessible, charged groups in the protein molecule. The fact that increasing the ionic strength of the eluting solvent detaches the enzyme from Seph-C_6-NH_2 could support this suggestion. On the other hand, the increased retention power of columns with longer hydrocarbon chains may increase due to increased hydrophobic interactions between the hydrocarbon chains and appropriately sized hydrophobic "pockets" in the protein molecule. In this context it should be noted that, on the average, the free energy ($-\Delta F$) which may be obtained by the transfer of a linear hydrocarbon chain from water to a nonpolar environment increases by approximately 0.8 kcal/mol for each methylene group which is added to the chain (Jencks, 1969). In systematic studies of alkylagaroses (Seph-C_n) it was shown (Er-el *et al.*, 1972; Shaltiel, 1974a,b) that the retention power of these columns for a given protein increases upon increasing the length of the hydrocarbon side chains in spite of the fact that these agaroses have no charged groups at the tip of their arms. These studies, together with the fact that detachment of proteins bound to Seph-C_n columns can be achieved by increasing the hydrophobicity of the eluting solvent (Shaltiel and Er-el, 1973; Hofstee and Otillio, 1973) and that at least in some cases elution cannot be achieved by increasing the ionic strength of the eluent (Shaltiel *et al.*, 1973), led to the conclusion that hydrophobic interactions play a key role during chromatography on hydrocarbon-coated agaroses (Er-el *et al.*, 1972; Shaltiel and Er-el, 1973). In some cases, retained proteins can be detached from Seph-C_n columns by increasing the ionic strength or altering the pH of the eluent. Yet when dealing with proteins, whose conformation and aggregation state depend on intramolecular ionic interactions, it is quite conceivable that ionic strength and pH may affect the structure of the protein so that there is a change in the size and availability of its hydrophobic "pockets."

In view of these and other observations (Er-el *et al.*, 1972; Shaltiel and Er-el, 1973; Yon, 1972; Hofstee and Otillio, 1973) one should consider the possibility that the commonly used ion exchangers which contain branched hydrocarbon chains (diethylaminoethyl groups) are in fact special cases of mixed ionic and hydrophobic adsorbents, and could be regarded as isolated members of larger families which have not yet been systematically investigated. This suggestion could account for some abnormalities in the binding of

proteins to ion exchangers on the "wrong" side of their isoelectric point (Himmelhoch, 1972).

An interesting observation reported in the present paper deals with a comparison between the ω-aminoalkylagarose chosen (Seph-C_6-NH_2) and DEAE-cellulose; Seph-C_6-NH_2 gave a better purification, removing protein impurities which were still present after passage through the DEAE-cellulose column. This is presumably due to the fact that in selecting Seph-C_6-NH_2 we adjusted the contribution of hydrophobic interactions to the optimal level.

Finally, this paper illustrates the usefulness of homologous series of ω-aminoalkylagaroses in protein purification as compared with affinity chromatography. It provides an example of how these two techniques can be combined to achieve efficient purification of an enzyme.

References

Axen, R., Porath, J., and Ernback, S. (1967), *Nature (London) 214,* 1302.

Cuatrecasas, P., and Anfinsen, C. B. (1972), *Methods Enzymol. 23,* 345.

Er-el, Z., Zaidenzaig, Y., and Shaltiel, S. (1972), *Biochem. Biophys. Res. Commun. 49,* 383.

Henderson, G. B., and Snell, E. E. (1973), *J. Biol. Chem. 248,* 1906.

Himmelhoch, S. R. (1972), *Methods Enzymol. 22,* 273.

Hofstee, B. H. J., and Otillio, N. F. (1973), *Biochem. Biophys. Res. Commun. 50,* 751.

Jencks, W. P. (1969), *in* Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill, p 401.

Shaltiel, S. (1974a), *in* Metabolic Interconversion of Enzymes—1973, Fischer, E. H., Krebs, E. G., Neurath, H., and Stadtman, E. R., Ed., Heidelberg Springer-Verlag, in press.

Shaltiel, S. (1974b), *Methods Enzymol. 34B* (in press).

Shaltiel, S., Ames, G. F., and Noel, K. D. (1973), *Arch. Biochem. Biophys. 159,* 174.

Shaltiel, S., and Er-el, Z. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 778.

Shaltiel, S., Henderson, G. B., and Snell, E. E. (1974), *Biochemistry 13,* 4330.

Yon, R. J. (1972), *Biochem. J. 126,* 765.

Electron Paramagnetic Resonance Studies of the Structure and Metal Ion Exchange Kinetics of Vanadyl(IV) Bovine Carbonic Anhydrase†

John J. Fitzgerald‡ and N. Dennis Chasteen*

ABSTRACT: Detailed electron paramagnetic resonance (epr) studies of powder, room temperature, and frozen solution samples of vanadyl(IV) bovine carbonic anhydrase B (VO^{2+}-BCA), in which VO^{2+} has been substituted for Zn^{2+} in the native enzyme, are reported. The modified enzyme is inactive toward *p*-nitrophenyl acetate. In solution, high and low pH forms of the metal site exist with a functional group(s) having an apparent pK_a = 7.1 involved. The functional group does not appear to be an OH^- group bound to the metal as has been suggested for the Zn^{2+} and Co^{2+} enzymes by other workers. Epr parameters suggest that the high pH form is structurally similar to that found in powder samples, *i.e.*, the VO^{2+} is coordinated to at least two imidazole groups of histidyl residues. In the low pH form, which does not exist in powder samples, the enzyme has undergone an important conformational rearrangement. One possible interpretation is an increase in the number of coordinating protein ligands at low pH. The stability constant for the VO^{2+}-BCA complex was measured as a function of pH and is approximately 10^{11} at pH 8.0. At pH 7.9 and 25° the "spontaneous" dissociation rate of the VO^{2+}-BCA complex, as measured by epr, follows first-order kinetics in [VO^{2+}-BCA] with a rate constant of 6.8×10^{-4} sec^{-1}. Under the same conditions, the Zn^{2+}-promoted VO^{2+} dissociation is first order in both [Zn^{2+}] and [VO^{2+}-BCA] with a second-order rate constant of 1.4 sec^{-1} M^{-1}. This study further demonstrates the applicability of VO^{2+} epr spectroscopy as a metal probe capable of providing structural and kinetic information about metal binding sites in proteins.

Electron paramagnetic resonance spectroscopy (epr)[1] yields information which is often difficult to obtain by other means. The method is particularly well suited for biological investigations because of its applicability to dilute aqueous samples. The analyses of epr spectra of polycrystalline vanadyl (VO^{2+}) insulin and carboxypeptidase A have demonstrated the utility of VO^{2+} epr measurements in probing metal binding sites in crystalline proteins (Chasteen *et al.*, 1973; DeKoch *et al.*, 1974). Use of this site-specific epr probe in correlating structural information such as that obtained by X-ray crystallography with *in vitro* room temperature solution studies is heretofore unexplored.

In the past, extrapolation of X-ray structural results to

† From the Department of Chemistry, University of New Hampshire, Durham, New Hampshire 03824. *Received March 15, 1974.* Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, and to the National Institute of General Medical Sciences (Grant GM 20194-02).

‡ Present address: Gillette Co., Boston, Mass. 02106.

[1] Abbreviations used are: epr, electron paramagnetic resonance; VO^{2+}-BCA, vanadyl(IV) bovine carbonic anhydrase B; NphOAc, *p*-nitrophenyl acetate.