AFFINITY CHROMATOGRAPHY OF CARBONIC ANHYDRASE

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Received 12 May 1972

1. Introduction

During the last years several examples have been presented showing how the covalent attachment of an enzyme inhibitor, cofactor, or substrate, to an inert matrix can provide an adsorbent useful for the selective purification of an enzyme from a complex mixture [1–7]. The enzyme carbonic anhydrase is strongly and specifically inhibited by aromatic and heterocyclic sulfonamides [8,9]. These inhibitors would be expected to be useful in designing biospecific adsorbents for the enzyme.

The present paper describes the chromatography of human and bacterial carbonic anhydrases on an adsorbent consisting of Sephadex or Sepharose to which sulfanilamide had been coupled. After adsorption of the enzyme to the column elution can be brought about in a predictable manner by agents that are known to act competitively with sulfonamides. The human erythrocyte enzyme, as described elsewhere [9], consists of a mixture of two forms that differ from each other in the structure of the binding site for inhibitors. This difference can be utilized to separate the two isoenzymes by selective displacement after adsorption on a chromatography column. Preliminary results of this investigation have been communicated elsewhere [4] and later Whitney reported similar experiments [10].

2. Materials and methods

Sulfanilamide was coupled to Sephadex G-150 and Sepharoses 2B, 4B, and 6B using the cyanogen bromide method described previously [1]. The degree of modification of the polymers was determined by sulfur analysis [11]. Using a reaction mixture of 2 g of sulfanilamide and 25 g (dry weight) of cyanogenbromide-activated Sephadex G-150 resulted in a product containing 13 mg (=75 μequiv.) of sulfonamide/g of dry polymer which corresponds to a yield in the coupling of 16%. Similarly, the modification of Sepharose 2B carried out with 2 g of sulfanilamide per 5 g of polymer gave 48 mg (= 280 μequiv.) of sulfonamide/g of dry polymer corresponding to a yield of 12%.

Forms B and C of the human erythrocyte carbonic anhydrase were purified as described elsewhere [12] but avoiding lyophilization for enzyme C. Bacterial carbonic anhydrase was derived from *Neisseria sicca*, strain 6021, as described by Adler et al. [13].

Carbonic anhydrase activity was assayed as the esterase reaction following the hydrolysis of *p*-nitrophenyl acetate [14] or by measuring the rate of the carbon dioxide hydration by the method of Wilbur and Anderson [15] as modified by Rickli et al. [16]

A histidine side chain in the active-site region of human carbonic anhydrase B was chemically modified by the specific reaction with bromoacetate described by Whitney et al. [14]. The reaction was stopped half-way, i.e. at a residual activity of 55%, by putting

the reaction mixture on a column containing Sephadex G-25. The product derived which contained 0.51 equivalents of carboxymethylhistidine was fractionated on sulfanilamide–Sephadex as described in the caption to fig. 2.

Column chromatographies were carried out under experimental conditions described in the captions to the figures. Flow-rates were usually kept constant by using a peristaltic pump and fractions were collected with a time-regulated fraction collector. Protein concentrations were determined spectrophotometrically at 280 nm or by the ninhydrin reaction using a calibration curve constructed from a standard solution of the carbonic anhydrase to be analyzed.

3. Results and discussion

Fig. 1 shows how human carbonic anhydrase B can be adsorbed to the sulfanilamide-Sephadex and displaced from the column by acetazolamide. Acetazolamide is a potent carbonic anhydrase inhibitor of the sulfonamide type [8] with a dissociation constant (K_I) for the enzyme–inhibitor complex of about 5×10^{-7} M at the pH of the experiment. Acetazolamide acts competitively with the sulfanilamide groups covalently fixed to the adsorbent and the enzyme is eluted from the column as an enzyme–acetazolamide complex. In fig. 1 as in subsequent figures a minor amount of protein runs unadsorbed through

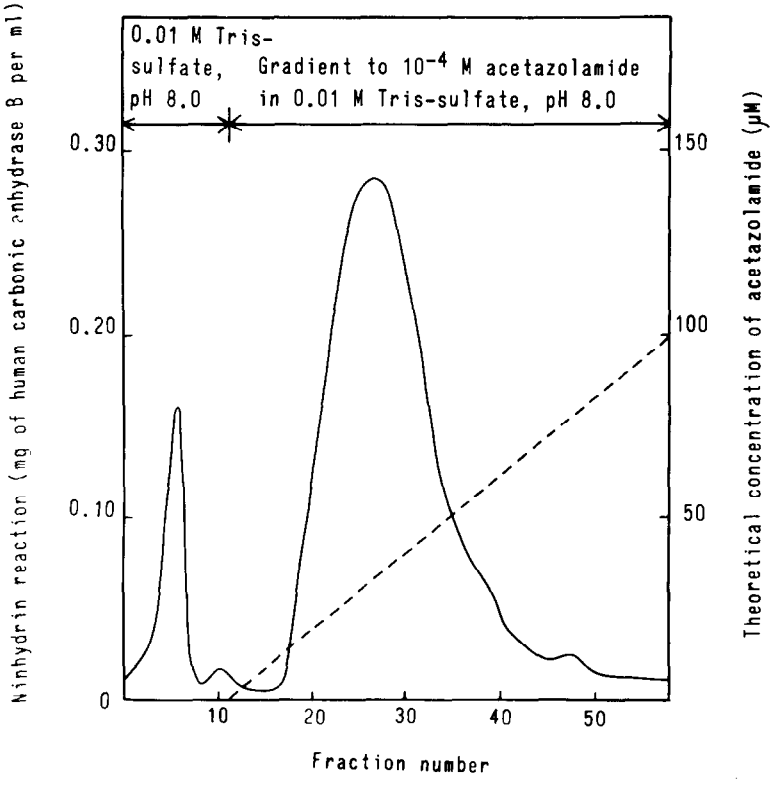

Fig. 1. Chromatography of human carbonic anhydrase B on sulfanilamide-Sephadex. 8.5 mg of enzyme in 0.01 M Tris-sulfate, pH 8.0, was applied to the column. The enzyme was displaced by the addition of an enzyme inhibitor of the sulfonamide type, acetazolamide, which was applied in the form of a linear gradient as indicated by the dotted line. Column dimensions: 120 × 10 mm. Temperature: 8–10°. Fraction volume: 2–3 ml. Flow rate: about 30 ml/hr. Due to the absorption in ultraviolet displayed by acetazolamide, the protein determination was carried out by the ninhydrin reaction. The material in the first peak did contain no or only trace amounts of enzyme activity.

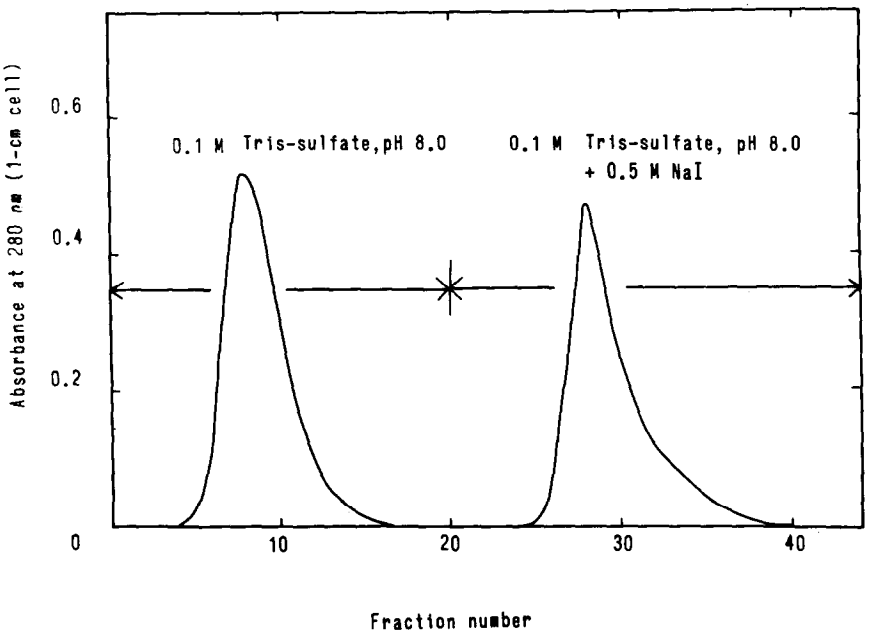

Fig. 2. Separation by affinity chromatography of native human carbonic anhydrase B from enzyme chemically modified in the active site region. The enzyme was modified by the specific reaction with bromoacetate described by Whitney et al [14], which leads to the carboxymethylation of a histidine side chain in the active-site region. The reaction was interrupted halfway giving a product containing 0.51 equivalents of carboxymethylhistidine. This product, dissolved in 4 ml of 0.1 M Tris-sulfate, pH 8.0, was applied to a column with sulfanilamide-Sephadex equilibrated with the same buffer. Elution was carried out as indicated in the figure. Column dimensions: 110 × 12 mm. Temperature: 8–10°. Fraction volume: about 2 ml. Flow rate: 15 ml/hr. Fractions containing protein were pooled and lyophilized. The first peak gave about 5.4 mg and contained 0.72 equivalents of carboxymethylhistidines while the second peak consisting of about 4.5 mg of protein contained only trace amounts (~0.03 equiv.) of modified histidine.

the column. This material has been ascribed to impurities in the enzyme preparation used in the experiments or, more likely, to carbonic anhydrase molecules having the inhibitor-binding site destroyed by denaturation.

The specificity in the interaction of carbonic anhydrase with the sulfanilamide-Sephadex is further illustrated by the experiment shown in fig. 2. Here a preparation of human carbonic anhydrase B partially altered in its active site by chemical modification has been chromatographed. Treatment of the human B enzyme with bromoacetate [14] or iodoacetate [17] leads to the insertion of a carboxymethyl group into the active site region. This group which becomes covalently attached to a histidine side chain is known to interfere with the binding of sulfonamides increasing their K_I values several orders of magnitude [18]. Fig. 2 illustrates how a preparation of human carbonic anhydrase B, carboxymethylated to about 50%, is separated on the sulfanilamide-Sephadex into two fractions differing in the content of modified histidine. The material retained on the column and subsequently eluted with 0.5 M NaI represents unmodified enzyme while the fraction running through the column has a content of carboxymethylhistidine higher than the material applied to the column.

The experiment in fig. 2 resembles that reported by Whitney [10] who purified human carbonic anhydrase B chemically modified in its active site with iodoacetamide [18]. The modified enzyme was prepared from the reaction mixture by passing through a column packed with Sepharose to which *p*-aminomethylbenzenesulfonamide had been coupled.

X-ray diffraction studies on human carbonic anhydrase C [19] show that the active site of the enzyme where the sulfonamide inhibitors bind, is

located in a crevice in the protein molecule. The zinc ion to which has been ascribed a central role in the catalytic reaction is located at the bottom of the cavity [9]. Sulfonamide inhibitors bind with their sulfonamide group oriented towards the metal ion. Monovalent anions like halide ions also inhibit the enzyme. They compete with the sulfonamide inhibitors as judged from investigations of the enzyme in solution [20]. X-ray diffraction studies on enzyme–inhibitor complexes also show that halide ions and sulfonamides have overlapping binding sites [21]. Halide ions would therefore be expected to displace carbonic anhydrase from the specific adsorbent. This prediction is validated by the results in fig. 3. Cyanate is another inhibitor competing with sulfonamide binding [22]. It binds several orders of magnitude more strongly to the enzyme than the halide ions. The use of cyanate as eluting agent is also demonstrated in fig. 3.

Human carbonic anhydrases B and C differ in the structure of their active site as is evident from dissimilarities in catalytic properties [23] and binding of sulfonamide inhibitors [24]. At pH around 8 the K_I values for sulfanilamide can be estimated to about 20×10^{-6} and 5×10^{-6} M, respectively, i.e. the inhibitor associates more weakly with enzyme B than with the C form. The halide ions, on the other hand, show stronger affinity to enzyme B than to the C form [9]. The differences between the two forms of the human carbonic anhydrase in inhibitor binding is reflected in the behaviour of the proteins on sulfanilamide-Sephadex (fig. 3 B and C). It can be utilized for separating the two proteins from each other in a single run as demonstrated in fig. 3D.

For both human carbonic anhydrases the binding strength of halide ions increases in the order $Cl^- < Br^- < I^-$ [9]. Fig. 3 B shows how human carbonic anhydrase B after adsorption to the column can be eluted by 0.1 M NaI. If NaCl is used instead

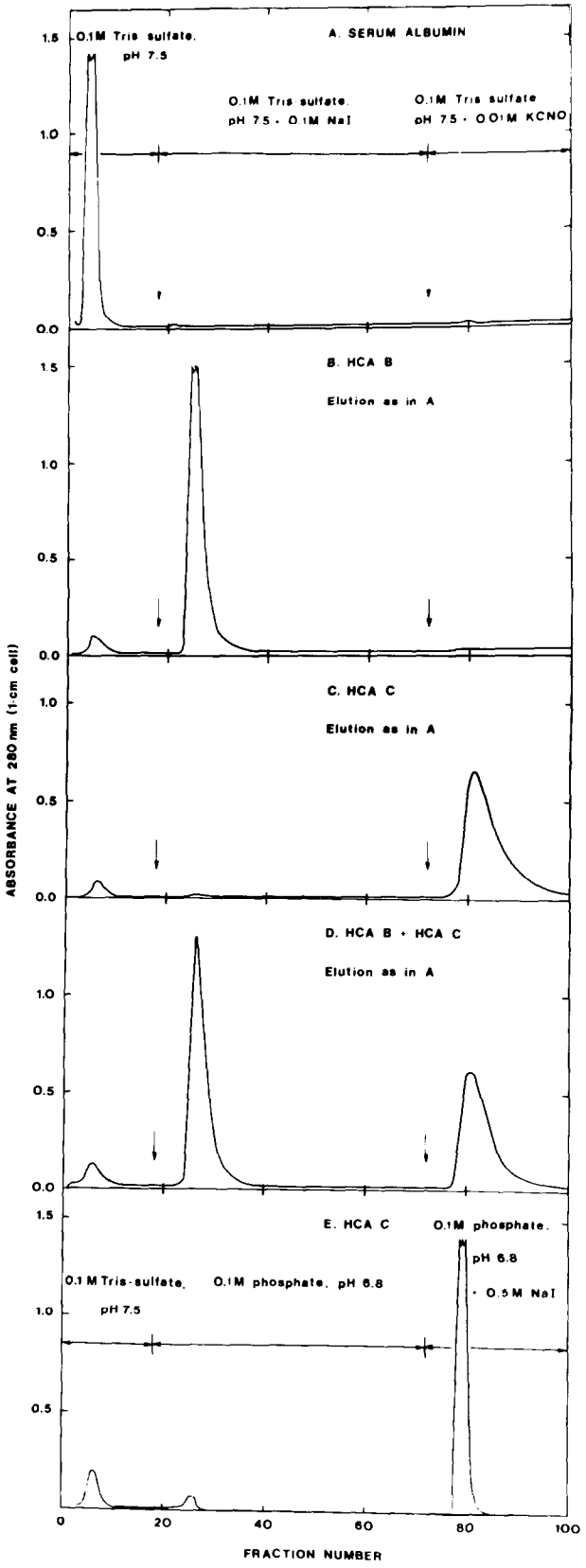

Fig. 3. Chromatography of human carbonic anhydrases B (HCA B) and C (HCA C) on sulfanilamide-Sephadex. Serum albumin was also chromatographed on the same column as an example of a protein without specific interaction with the adsorbent. Column dimensions: 90 × 12 mm. Temperature: 8–10°. Fraction volume: 2 ml. Flow rate (kept constant with a peristaltic pump): 6.8 ml/hr. 5–10 mg of protein was applied to the column. Stepwise elution was carried out as specified in the diagrams.

the concentration required for displacing the protein from the column should be higher. Successful elutions have been carried out with 2 M NaCl. The binding of halide ions increases with decreasing pH while the strength of sulfonamide binding in the same pH range becomes weaker [9]. This phenomenon would imply that in chromatography of carbonic anhydrase on sulfonamide-containing specific adsorbents the control of pH would be of importance for reproducibility. Alternatively, controlled changes pf pH could be utilized for the purpose of separation. Fig. 3 C and D show how NaI at pH 7.5 is a suitable eluting agent for enzyme B while the C enzyme remains attached to the column. By lowering the pH, NaI can also be used to displace the C enzyme from the adsorbent as demonstrated in fig. 3 E.

The utilization of the specific adsorbent for preparative purposes is illustrated in figs. 4 and 5. Fig. 4 shows the purification of a bacterial carbonic anhydrase from *Neisseria sicca*. This enzyme resembles human carbonic anhydrase C in sulfonamide inhibition [25] and fig. 4 shows how the bacterial protein can be purified from a crude extract by adsorption and subsequent elution from the column under conditions similar to those found useful in the case of the human enzymes given in fig. 3. Fig. 5 illustrates the purification of the two human forms from hemolysate of red blood cells using the same experimental conditions as used in the chromatography of the purified enzyme forms (fig. 3). Another example reported elsewhere [26] is the purification of a carbonic anhydrase from human kidney that appears to be indistinguishable from the C form isolated from erythrocytes.

The behaviour of carbonic anhydrases on sulfanilamide-Sephadex described above can clearly be accounted for in terms of active-site-specific binding as determining adsorption and desorption of the enzyme. The experimental conditions utilize only part of the vast knowledge about inhibitor binding to carbonic anhydrase [8,9]. Several other possibilities remain to be exploited, in particular there would be a great choice of sulfonamide inhibitors for producing adsorbents [8]. In addition to the investigations with sulfanilamide-Sephadex described above, a few experiments were also carried out with sulfanilamide cuopled to Sepharose. The results obtained were less reproducible than for the Sephadex derivative. As

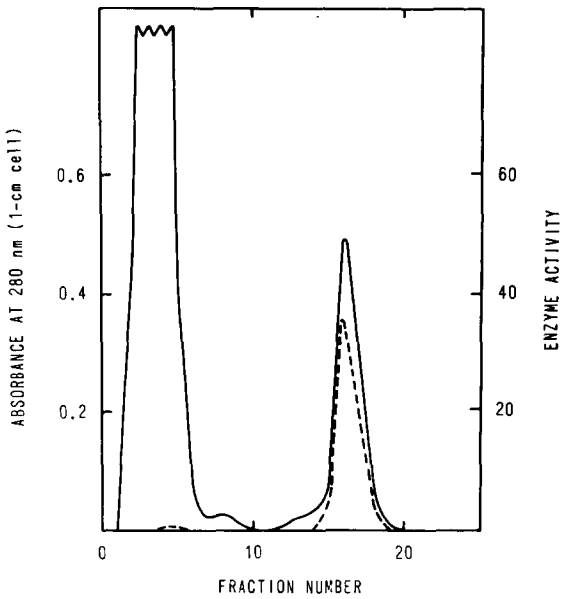

Fig. 4. Purification of bacterial carbonic anhydrase from *Neisseria sicca* on sulfanilamide-Sephadex. A crude extract of enzyme from step 2 in the procedure of Adler et al. [13] was dialyzed against 0.1 M Tris-sulfate, pH 7.5, and applied to the column, preequilibrated with the same buffer. The column was washed with buffer and the enzyme eluted with 0.1 M Tris-sulfate, pH 7.5 + 0.01 M KCNO. Enzyme activity (– – – – –) and protein concentration (———) were determined as described in Materials and methods. Column dimensions: 100 × 12 mm. Room temperature. Fraction volume: 3 ml. Flow rate: 18 ml/hr.

will be reported elsewhere (Sundberg and Wåhlstrand) technical improvements can be made by coupling sulfanilamide to a spacer incorporated on cross-linked agarose [27].

Acknowledgement

We wish to thank Professor Bo G. Malmström for valuable suggestions and encouragement. Financial support from the Swedish Medical and Natural Science Research Councils, the Institute of General Medical Science U.S. Public Health Service (GM 12280-06), and the Swedish Board for Technical Development is gratefully acknowledged.

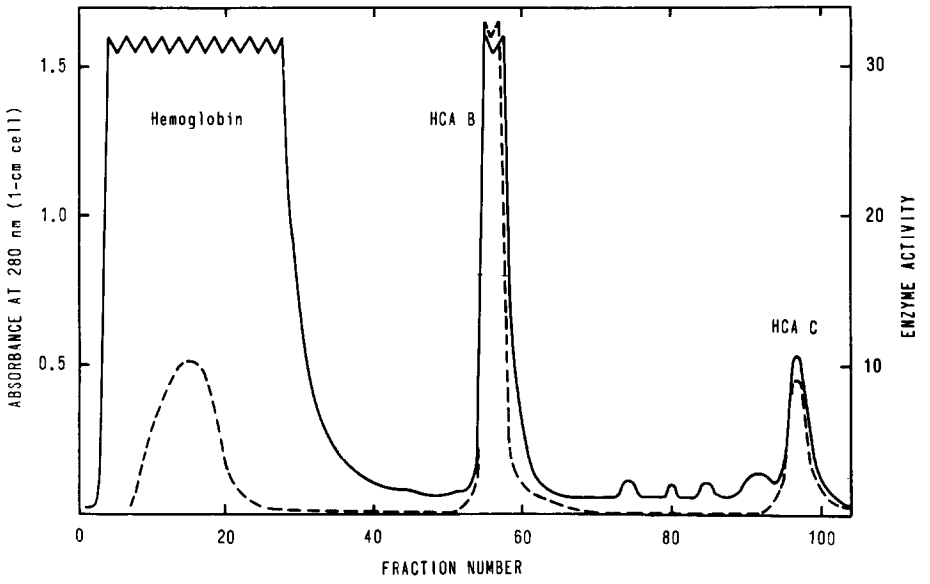

Fig. 5. Chromatography of hemolysate on sulfanilamide-Sephadex. Human erythrocytes were washed with 0.9% NaCl and hemolyzed with distilled water. The hemolysate was very carefully dialyzed against 0.1 M Tris-sulfate, pH 7.5, and centrifuged. 25 ml were applied to the column. Washing with buffer brought the hemoglobin through the column together with a certain amount of enzymic activity. Carbonic anhydrases B (HCA B) and C (HCA C) were subsequently eluted with 0.1 M NaI and 0.01 M KCNO in Tris-sulfate, pH 7.5 as in the chromatograms shown in fig. 3. Enzymic activity (- - - - - - -) and protein concentration (————) were determined as described in Materials and methods. Column dimensions: 150 × 20 mm. Room temperature. Fract. vol. 4–5 ml.

References

[1] R. Axén, J. Porath and S. Ernback, Nature 214 (1967) 1302.
[2] J. Porath, R. Axén and S. Ernback, Nature 215 (1967) 1491.
[3] J. Porath, Nature 218 (1968) 834.
[4] J. Porath and L. Sundberg, in: Protides of the Biological Fluids, Proceedings of the 18th Colloquium, Bruges 1970, ed. H. Peeters (Pergamon Press, Oxford and New York, 1971) p. 401.
[5] P. Cuatrecasas and C.B. Anfinsen, Ann. Rev. Biochem. 40 (1971) 259.
[6] P. Cuatrecasas and C.B. Anfinsen, in: Methods in Enzymology, Vol. XXII, ed. W. Jacoby (Academic Press, New York and London, 1971) p. 345.
[7] J. Porath and L. Sundberg, in: The Chemistry of Biosurfaces, Vol. II, ed. M. Hair (Dekker, New York, 1972) 0. 633.
[8] T.H. Maren, Physiol. Rev. 47 (1967) 595.
[9] S. Lindskog, L.E. Henderson, K.K. Kannan, A. Liljas, P.O. Nyman and B. Strandberg, in: The Enzymes, Vol. 5, ed. P.D. Boyer (Academic Press, New York and London, 1971) p. 587.
[10] P.L. Whitney, Federation Proc. 30 (1971), No. 3, p. 1291, Abstract 1389.
[11] L. Gustavsson, Talanta 4 (1960) 227.
[12] P.O. Nyman, L. Strid and G. Westermark, European J. Biochem. 6 (1968) 172.
[13] L. Adler, J. Brundell, S.O. Falkbring and P.O. Nyman, Biochim. Biophys. Acta, submitted.
[14] P.L. Whitney, P.O. Nyman and B.G. Malmström, J. Biol. Chem. 242 (1967) 4212.
[15] K.M. Wilbur and N.G. Anderson, J. Biol. Chem. 176 (1948) 147.
[16] E.E. Rickli, S.A.A. Ghazanfar, G.H. Gibbons and J.T. Edsall, J. Biol. Chem. 239 (1964) 1065.
[17] S.L. Bradbury, J. Biol. Chem. 244 (1969) 2002 and 2010.
[18] P.L. Whitney, European J. Biochem. 16 (1970) 126.
[19] A. Liljas, K.K. Kannan, P.C. Bergsten, I. Waara, K. Fridborg, B. Strandberg, U. Carlbom, L. Järup, S. Lövgren and M. Petef, Nature 235 (1972) 131.
[20] P.W. Taylor and A.S.W. Burgen, Biochemistry 10 (1971) 3859.
[21] A. Liljas, Crystal Structure Studies of Human Erythrocyte Carbonic Anhydrase C at High Resolution. Acta Universitatis Upsaliensis, Uppsala Dissertations from the

Faculty of Science 3 (1971) (Almquist och Wiksell, Gamla Brogatan 26, S-101 20 Stockholm, Sweden).
[22] J.E. Coleman, J. Biol. Chem. 242 (1967) 5212.
[23] R.G. Khalifah, J. Biol. Chem. 246 (1971) 2561.
[24] P.W. Taylor, R.W. King and A.S.V. Burgen, Biochemistry 9 (1970) 2638.
[25] J. Brundell, S.O. Falkbring and P.O. Nyman, Biochim. Biophys. Acta, submitted.
[26] S.O. Falkbring, P.O. Nyman, T. Wåhlstrand and P. Wistrand, Abstract XXV, Scand. Pharmacol, Meeting, Acta Pharmacol et Toxicol. 29 (1971) suppl. 4, p. 61.
[27] J. Porath, J.C. Janson and T. Låås, J. Chromatog. 60 (1971) 167.