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Purification and characterization of a high-M_r carbonic anhydrase from sheep parotid gland

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Approximately half the carbonic anhydrase activity of sheep parotid-gland homogenate is derived from a high-M_r protein [Fernley, Wright & Coghlan (1979) FEBS Lett. **105**, 299–302]. This enzyme has now been purified to homogeneity, and its properties were compared with those of the well-characterized sheep carbonic anhydrase II. The protein has an apparent M_r of 540000 as measured by gel filtration under non-denaturing conditions and an apparent subunit M_r of 45000 as measured by SDS/polyacrylamide-gel electrophoresis. After deglycosylation with the enzyme *N*-glycanase the protein migrates with an apparent M_r of 36000 on SDS/polyacrylamide-gel electrophoresis. The CO_2-hydrating activity was 340 units/mg compared with 488 units/mg for sheep carbonic anhydrase II measured under identical conditions. This enzyme does not, however, hydrolyse *p*-nitrophenyl acetate. The enzyme contains 0.8 g-atom of zinc/mol of protein subunit. The peptide maps of the two carbonic anhydrases differ significantly from one another, indicating they are not related closely structurally. Unlike the carbonic anhydrase II isoenzyme, which has a blocked *N*-terminus, the high-M_r enzyme has a free glycine residue at its *N*-terminus.

INTRODUCTION

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1), which catalyses the reversible hydration of CO_2 [1], has been isolated from a wide range of species and tissues [2,3,4]. From mammalian tissues, three isoenzymes of carbonic anhydrase have been well characterized: carbonic anhydrase I, a low-activity isoenzyme, which occurs primarily in erythrocytes, carbonic anhydrase II, the high-activity isomer, which has a wide tissue distribution, and carbonic anhydrase III, which is present mainly in red skeletal muscle [5]. The three isoenzymes are the products of three different genes [6]. All are monomeric proteins with an M_r of about 30000 containing one zinc atom per molecule. Another group of carbonic anhydrases, which are not as well characterized, are the membrane-bound enzymes. They appear to differ from the other isoenzymes in a number of properties. These membrane-bound enzymes have been classified as carbonic anhydrase IV. These detergent-solubilized proteins have been difficult to purify, which explains the paucity of data relating to them [7]. However, it is known that they have higher M_r values than the other isoenzymes. For example, human renal carbonic anhydrase IV has an M_r of 68000 [7] and bovine lung carbonic anhydrase IV an M_r of 52000 [8]. At least part of this appears to be due to the presence of carbohydrate. These isoenzymes also have amino acid compositions different from those of the other isoenzymes.

In 1979 we reported the presence of a high-M_r carbonic anhydrase in the parotid gland and saliva of sheep [9]. This enzyme accounted for about 40% of the total carbonic anhydrase activity of the parotid gland, the rest being due to the carbonic anhydrase II isoenzyme. The apparent M_r of this enzyme was estimated to be 240000.

The aim of the present study was to isolate and characterize this high-M_r carbonic anhydrase. A preliminary report on the purification of this enzyme has been presented [10]. Another group [11,12] has reported the presence of a similar carbonic anhydrase in rat salivary glands and in saliva.

EXPERIMENTAL

Materials

Activated carboxyhexyl- (CH-)Sepharose 4B, Sepharose 6B, Sephadex G-50 (superfine grade), Sephadex G-75 (fine grade), DEAE-Sephacel, wheat-germ lectin–Sepharose 6MB, low-M_r electrophoresis calibration kit, high-M_r gel-filtration calibration kit and *N*-acetyl-D-glucosamine were obtained from Pharmacia Fine Chemicals. Ultra-pure Tris base and Tris hydrochloride were purchased from Schwarz–Mann. CNBr was from Pierce Chemical Co. SDS, acrylamide, bisacrylamide and *NNN'N'*-tetramethylethylenediamine were from Bio-Rad Laboratories. *N*-Glycanase was from Genzyme Corp. Reagents used for protein sequencing were from Applied Biosystems. Iodoacetic acid was recrystallized from cold light petroleum and stored in a desiccator at −20 °C. Other chemicals used were of analytical grade.

Purification of carbonic anhydrases

Carbonic anhydrase affinity resin was made by coupling 1 g (4.5 mmol) of *p*-aminomethylbenzenesulphonamide hydrochloride (Sigma Chemical Co.) to 15 g of activated CH-Sepharose 4B by using the procedure recommended by the manufacturer.

Parotid salivary glands were excised from Merino and Merino cross-bred sheep immediately after death. The glands were trimmed of excess fat and either frozen and stored at −20 °C or homogenized in 50 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA and 0.5 mM-phenylmethanesulphonyl fluoride. All puri-

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fication steps were carried out at 4 °C. The homogenate was centrifuged (20000 *g* for 20 min) and the supernatant filtered through glass-wool to remove any solid fat. An equal volume of a saturated $(NH_4)_2SO_4$ solution was added to the supernatant and the mixture was stirred for 1 h. The suspension was centrifuged (10000 *g* for 15 min) and the precipitate taken up in minimal 0.1 M-NH_4HCO_3 and dialysed against this buffer overnight. After centrifugation to remove any particulate matter, the supernatant was mixed with 40 ml of the sulphonamide affinity resin and allowed to bind for 1 h. The resin was poured into a column and washed extensively with 0.1 M-NH_4HCO_3. More inactive protein was removed by washing the column with 0.2 M-NaI in 0.1 M-NH_4HCO_3. Carbonic anhydrases were eluted from the column with 0.1 M-KCN in 0.1 M-NH_4HCO_3. These fractions were concentrated by ultrafiltration and loaded on to a column of wheat-germ lectin–Sepharose equilibrated in 50 mM-sodium phosphate buffer, pH 7.4, containing 0.2 M-NaCl. After unbound protein was washed from the resin, the carbonic anhydrase was eluted by a 100 mg/ml solution of *N*-acetyl-D-glucosamine in 50 mM-sodium phosphate buffer, pH 7.4, containing 0.2 M-NaCl. The eluted peak was chromatographed on a column of Sepharose 6B in 0.1 M-NH_4HCO_3.

Carbonic anhydrase II isoenzyme was purified from parotid homogenate by affinity chromatography as described for the high-M_r enzyme, followed by gel filtration on Sephadex G-75 and ion-exchange chromatography on DEAE-Sephacel in 50 mM-Tris buffer, pH 8.7. Carbonic anhydrase activity was eluted from this column with a linear salt gradient (0–100 mM-NaCl).

Carbonic anhydrase assay

The CO_2-hydrating activity of the preparations was measured as previously described [9], or activity was measured by the hydrolysis of *p*-nitrophenyl acetate [13].

Polyacrylamide-gel electrophoresis

Gel electrophoresis to determine protein purity and subunit M_r was performed on a 7.5–15%-gradient polyacrylamide gel in the presence of 0.1% SDS. Proteins of known M_r were run adjacent to the enzyme. This procedure was repeated four times, and the mean R_F values were plotted against $\log M_r$.

Removal of carbohydrate

High-M_r carbonic anhydrase (50 μg) was denatured by boiling (3 min) in 10 μl of 0.5% SDS/0.1 M-2-mercaptoethanol. To this was then added 10.8 μl of 0.55 M-sodium phosphate buffer, pH 8.6, 3.0 μl of 100 mM-1,10-phenanthroline (in methanol), 5.0 μl of 7.5% Nonidet P40 and 1.2 μl (0.34 unit) of *N*-glycanase (glycopeptide *N*-glycosidase F). This was incubated overnight at 37 °C and run on a polyacrylamide gel as described above. A control sample of enzyme was treated as above except that *N*-glycanase was omitted. Samples of carbonic anhydrase II were treated similarly with and without *N*-glycanase and run on the gel.

Native M_r

The M_r of the native enzyme was determined by the method of Andrews [14]. Standard proteins were run on a column of Sepharose 6B equilibrated in 0.1 M-NH_4HCO_3 and the elution volumes were measured. The standard proteins and the carbonic anhydrase were run six times each on the column. The means of the elution volumes were plotted against the $\log M_r$ values of the standard proteins.

Immunization protocol

Purified high-M_r carbonic anhydrase (1 mg) in 1 ml of 0.9% NaCl was emulsified with 1 ml of Freund's complete adjuvant. Two male New Zealand rabbits were injected subcutaneously with 1 ml of the mixture. Booster injections (0.5 mg of protein per rabbit) in Freund's incomplete adjuvant were given at 4-week intervals over a 6-month period. Bleeds were taken from the ear vein of the rabbits 6 weeks after the first injection and then every 4 weeks. A similar protocol was followed with another two rabbits to raise antibodies against carbonic anhydrase II.

Amino acid analysis

Samples were hydrolysed in 6 M-HCl *in vacuo* in sealed tubes containing 5 μl of phenol and 2 μl of 2-mercaptoethanol for 24, 48 and 72 h and analysed on a Beckman 6300 amino acid analyser.

Sialic acid analysis

Samples of carbonic anhydrase for sialic acid analysis were treated with 0.2 M-H_2SO_4 and heated at 80 °C for 1 h to release the sialic acid. This sugar was then determined by the method of Hammond & Papermaster [15].

Metal analysis

A column (1.2 cm × 25 cm) of Sephadex G-50 (superfine grade) was thoroughly equilibrated in a metal-ion-free buffer (50 mM-Tris, pH 8.0) made from Ultra-pure Tris base and Tris hydrochloride. Samples of both high-M_r and low-M_r parotid carbonic anhydrases were passed through the column and the peaks were collected. Buffer passed through the column was used as a blank. Zinc analyses were carried out on these samples on a

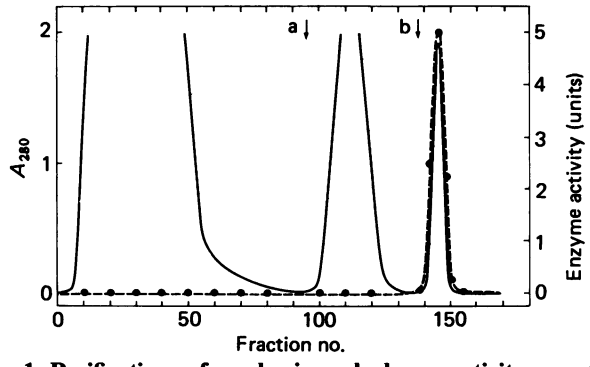

Fig. 1. Purification of carbonic anhydrase activity on the sulphonamide affinity column

The preparation of the column and sample were as described in the Experimental section. The parotid homogenate was loaded on to the column (40 cm × 2.5 cm), and unbound protein was washed through with 0.1 M-NH_4HCO_3. 0.2 M-NaI in the bicarbonate buffer was then applied to the column (a). Carbonic anhydrase activity (●--●) was eluted with 0.1 M-KCN in the bicarbonate buffer (b). ——, A_{280}. The column was run at 36 ml/h, and 20 min fractions were collected.

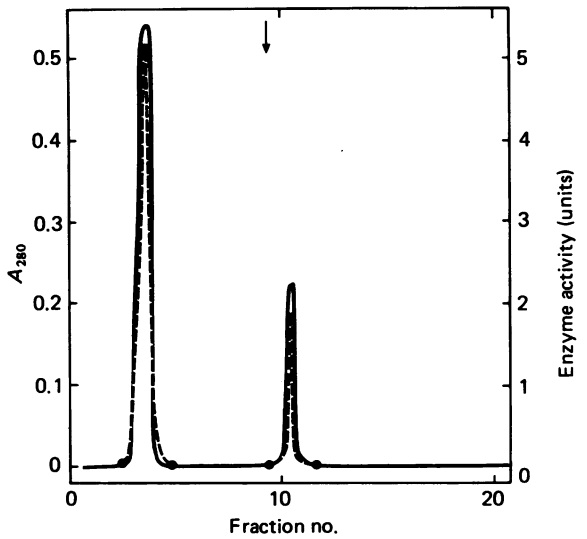

Fig. 2. Purification of carbonic anhydrase on wheat-germ lectin–Sepharose

The KCN-eluted protein from the sulphonamide affinity column was concentrated to about 5 ml and loaded on to a column (2.5 cm × 5 cm) of wheat-germ lectin–Sepharose in 50 mM-phosphate buffer, pH 7.4, containing 0.2 M-NaCl. After unbound protein was washed from the column with starting buffer, carbonic anhydrase activity (●--●) was eluted from the resin with 100 mg of *N*-acetyl-D-glucosamine/ml in the starting buffer. ——, A_{280}. The column was run at 1 ml/min, and 5 min fractions were collected.

Perkin–Elmer 272 atomic absorption spectrophotometer. Protein in the samples was measured by a modified Lowry procedure [16], with bovine serum albumin (ICN Pharmaceuticals) as standard.

Peptide mapping

Samples (2 mg each) of high-M_r and isoenzyme II carbonic anhydrases were reduced and carboxymethylated [17]. The modified proteins were then cleaved with 6 mg (each) of CNBr in 70% (w/v) formic acid overnight under an N_2 atmosphere [18]. The samples were diluted 10-fold with water and freeze-dried. The peptides were separated by using a Waters h.p.l.c. system equipped with a μBondapak C_{18} reversed-phase column. The solvent was 0.1% (v/v) trifluoroacetic acid, and a linear gradient in acetonitrile from 5 to 100% (v/v) at 1 ml/min was applied to elute the peptides.

N-Terminal analysis

Reduced and carboxymethylated high-M_r carbonic anhydrase (100 pmol) was sequenced twice on an Applied Biosystems 470A protein sequencer with the on-line 120A phenylthiohydantoin analyser. The glass-fibre disc was loaded with 3 mg of Polybrene as carrier and precycled three times. Protein was then loaded on to the disc for sequence analysis.

RESULTS

Purification of carbonic anhydrase

Pure high-M_r carbonic anhydrase was obtained by a simple four-step procedure. The purification involved $(NH_4)_2SO_4$ precipitation, affinity chromatography on sulphonamide–Sepharose, wheat-germ lectin affinity chromatography and gel filtration on Sepharose 6B (Figs. 1, 2 and 3). A purification Table has not been included because both carbonic anhydrases are present at all stages of purification except the final two. However, this procedure yields about 1 mg of pure enzyme from a pair of parotid glands (about 20 g wet wt.). The well-characterized carbonic anhydrase II isoenzymes could be

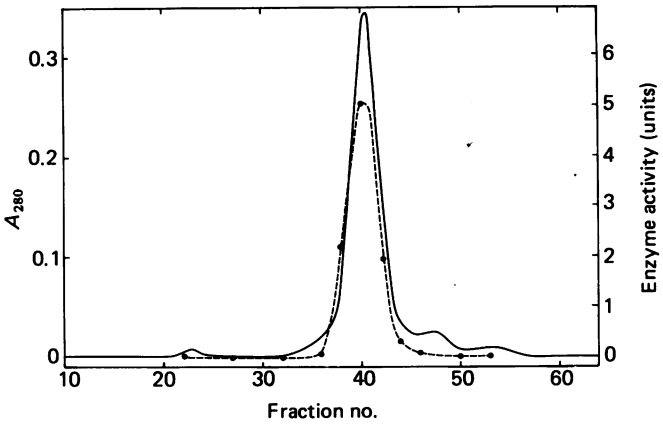

Fig. 3. Purification of high-M_r carbonic anhydrase by gel filtration on Sepharose 6B

Protein eluted from the lectin affinity column was concentrated by ultrafiltration to 3 ml and loaded on to a column (1.6 cm × 90 cm) of Sepharose 6B in 0.1 M-NH_4HCO_3. ●--●, Enzyme activity; ——, A_{280}. The column was run at 12 ml/h, and 30 min fractions were collected.

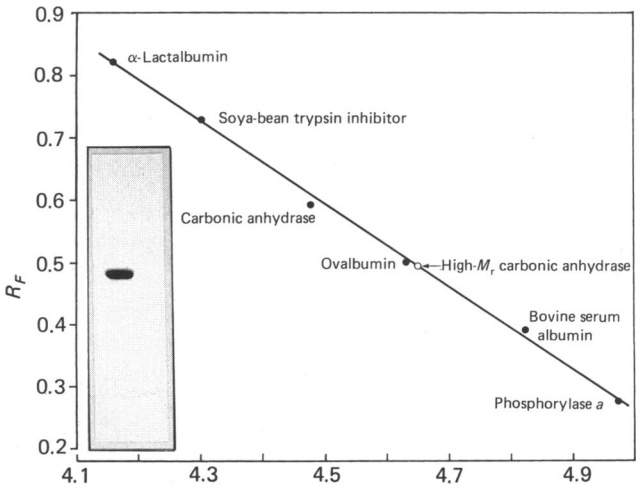

Fig. 4. Purity and subunit size of the high-M_r carbonic anhydrase

A 50 μg portion of purified high-M_r carbonic anhydrase was boiled in 0.1% SDS/0.5% 2-mercaptoethanol for 2 min, then run on a 7.5–15%-gradient polyacrylamide gel (20 cm × 20 cm × 0.15 cm) in the presence of 0.1% SDS. M_r-marker proteins were run with the sample. The gel was fixed and stained in 12.5% trichloroacetic acid/0.1% Coomassie blue. The marker proteins were phosphorylase *a* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase II (M_r 30000), soya-bean trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14400). The mobility of the proteins is plotted against the log M_r value.

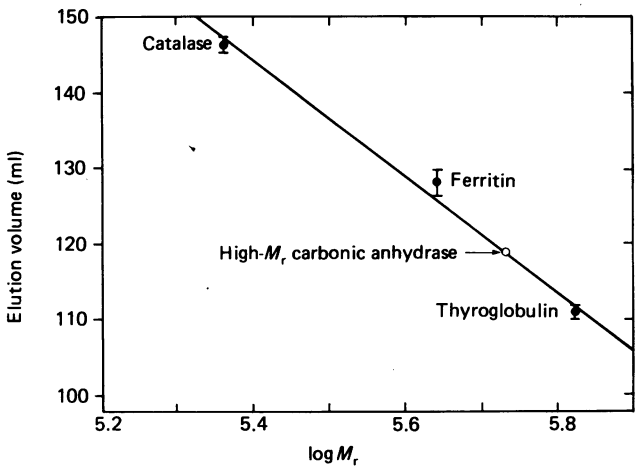

Fig. 5. Plot of log M_r versus elution volume of proteins as obtained from Sepharose 6B chromatography

Standard proteins and purified high-M_r carbonic anhydrase were chromatographed on a 1.2 cm × 98 cm column of Sepharose 6B in 0.1 M-NH_4HCO_3 and their elution volumes were measured. The marker proteins were thyroglobulin (M_r 669000), ferritin (M_r 440000) and catalase (M_r 232000). The ○ symbol indicates the elution volume of high-M_r carbonic anhydrase.

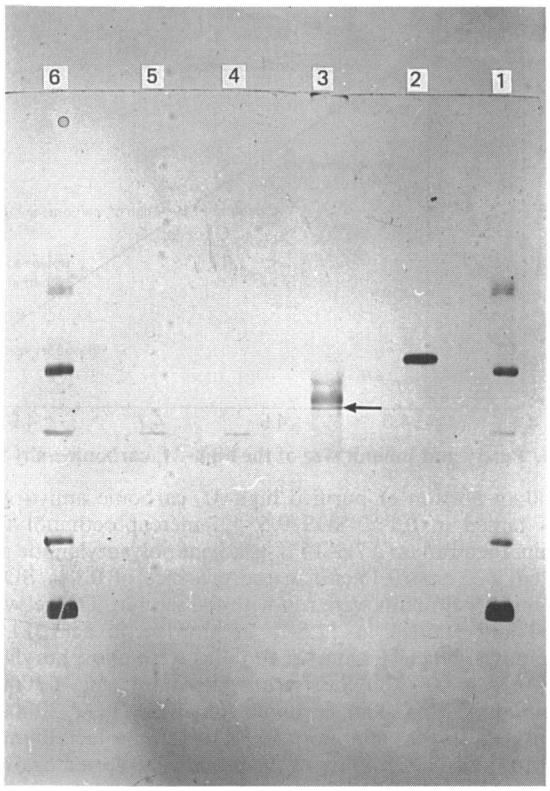

Fig. 6. Effect of *N*-glycanase on the subunit M_r of the high-M_r carbonic anhydrase

The protein was treated with *N*-glycanase as described in the Experimental section and run on a gradient polyacrylamide gel in the presence of SDS as described for Fig. 4. Lane 1, M_r markers; lane 2, untreated high-M_r carbonic anhydrase; lane 3, *N*-glycanase-treated high-M_r carbonic anhydrase; lane 4, control carbonic anhydrase II; lane 5, *N*-glycanase-treated carbonic anhydrase II; lane 6, M_r markers.

isolated by use of the same affinity resin, gel filtration on Sephadex G-75 and ion-exchange chromatography on DEAE-Sephacel (results not shown). This material was used for comparative purposes.

The high-M_r carbonic anhydrase appeared to be homogeneous when analysed by SDS/polyacrylamide-gel electrophoresis in the presence (or absence) of reducing agents (Fig. 4). This enzyme did not show any activity when *p*-nitrophenyl acetate was used as a substrate, but its CO_2-hydrating activity was 340 units/mg compared with 488 units/mg for sheep carbonic anhydrase II measured under identical conditions.

M_r of parotid carbonic anydrase

On a column of Sepahrose 6B equilibrated in 0.1 M-NH_4HCO_3, the purified enzyme was eluted between thyroglobulin (M_r 669000) and ferritin (M_r 440000) with an apparent M_r of 540000 ± 9000 (n = 6) (Fig. 5). When treated with SDS with or without reducing agent (2-mercaptoethanol), and run on a 7.5–15%-gradient polyacrylamide gel in the presence of 0.1% SDS the apparent subunit M_r was calculated to be 45000 ± 2000 (n = 5) (Fig. 4). The apparent M_r of the protein treated with *N*-glycanase was 36100 ± 620 (n = 4) (Fig. 6).

Antibodies

Antibodies raised against either carbonic anhydrase showed no cross-reactivity with the other enzyme (Fig. 7).

Amino acid and sialic acid analysis

The amino acid composition of high-M_r carbonic anhydrase is compared with the compositions of sheep carbonic anhydrase II and the enzyme isolated from rat saliva by Feldstein & Silverman [12] (Table 1). The sialic acid content was found to be 0.93 ± 0.28 (n = 4) residue/subunit.

Zinc analysis

Analysis of the zinc content of preparations of carbonic anhydrase by atomic absorption spectrometry gave a value of 0.8 g-atom of zinc/mol of the protein subunit

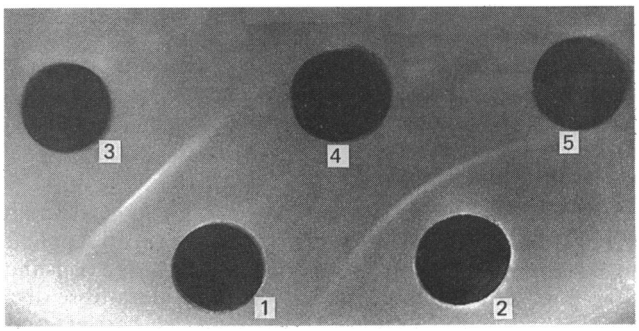

Fig. 7. Agar-gel diffusion of antibodies against high-M_r carbonic anhydrase and carbonic anhydrase II

Well 1 contained antiserum from a rabbit immunized against high-M_r carbonic anhydrase. Well 2 contained antiserum from a rabbit immunized against carbonic anhydrase II. Well 4 contained 10 μg of carbonic anhydrase II, and wells 3 and 5 contained 10 μg of high-M_r carbonic anhydrase. Diffusion was allowed to occur at room temperature for 24 h.

Table 1. Amino acid compositions of carbonic anhydrases

Amino acid	Amino acid composition (residues/100 residues)			
	Sheep parotid carbonic anhydrase (present study)	Sheep carbonic anhydrase II (present study)	Sheep carbonic anhydrase II (Tanis & Tashian [19])	Rat saliva carbonic anhydrase (Feldstein & Silverman [12])
Aspartic acid	10.3	12.6	12.6	11.9
Threonine*	6.7	3.9	4.2	7.0
Serine*	7.7	7.0	6.8	9.0
Glutamic acid	12.8	9.3	9.5	15.4
Proline	4.4	7.7	7.6	4.5
Half-cystine†	0.8	0	0	0.8
Glycine	6.7	7.0	6.8	7.4
Alanine	4.6	7.3	7.6	4.2
Valine‡	5.6	8.1	8.4	6.4
Methionine	2.3	1.2	1.1	3.1
Isoleucine‡	3.8	1.2	1.1	3.4
Leucine	10.1	10.0	9.9	7.0
Tyrosine	6.1	2.7	2.7	4.6
Phenylalanine	2.8	5.0	4.6	2.7
Histidine	4.1	4.2	4.2	4.9
Lysine	5.3	7.0	6.8	4.4
Arginine	4.6	3.5	3.8	5.6
Tryptophan§	1.3	2.3	2.3	0.5

* Values obtained by extrapolation to 0 h hydrolysis.
† Determined as cysteic acid after oxidation by performic acid [20].
‡ Value at 72 h hydrolysis used.
§ Determined spectrophotometrically [21].

compared with a value of 1.04 g-atom of zinc/mol for carbonic anhydrase II under identical conditions.

Peptide mapping and *N*-terminal analysis

The CNBr-cleavage peptide maps of the two carbonic anhydrases are shown in Fig. 8, and these appear to be quite different from one another. A glycine residue was found for the high-M_r enzyme on *N*-terminal analysis. An average initial yield of 66 pmol of glycine was found for the 100 pmol of protein sequenced.

DISCUSSION

An unusual carbonic anhydrase was found in the parotid salivary glands of the sheep and a method for its purification to homogeneity is described. The apparent M_r of the purified enzyme determined by gel filtration was 540000, although the accuracy of this value is unknown due to the anomolous behaviour of glycoproteins during gel filtration. This is approximately double the value estimated earlier [9], also by gel filtration. In those experiments, parotid homogenate supernatant was used rather than the purified enzyme. It appears that the enzyme has undergone aggregation during purification. Most of the well-characterized mammalian carbonic anhydrases have M_r values of 30000 and show no tendency to aggregate, although the membrane-bound carbonic anhydrases do have higher M_r values. These enzymes are solubilized only by treatment with detergent, whereas the sheep parotid enzyme is soluble. Bovine lung carbonic anhydrase IV is eluted from gel-filtration columns with an apparent M_r of about 100000 and migrates on SDS/polyacrylamide-gel electrophoresis with an apparent M_r of 52000 [8]. The human renal membrane carbonic anhydrase IV migrates on SDS/polyacrylamide-gel electrophoresis with an apparent M_r of 68000 [7]. The rat saliva enzyme has the same subunit M_r (46000) as this parotid enzyme [12]. The native M_r of this enzyme was not reported. All these enzymes have been shown to contain carbohydrate, which would affect the estimation of their M_r values by these methods. However, it does appear that the parotid carbonic anhydrase occurs as an oligomer in the native state.

The amino acid composition of the high-M_r carbonic anhydrase differs significantly from that of the carbonic anhydrase II enzyme. Again it appears most closely related to the carbonic anhydrase IV enzymes, with less proline and lysine and more glutamic acid/glutamine, methionine and arginine than the carbonic anhydrase II isoenzyme. Also, it contains cysteine, an amino acid absent from sheep carbonic anhydrase II, but present in the membrane-bound enzymes.

The high-M_r enzyme contains one residue of sialic acid/subunit. It also binds to wheat-germ lectin–Sepharose, indicating that it probably contains glucosamine residues. This sugar has been found in the bovine lung membrane-bound carbonic anhydrase [8]. The enzyme also binds strongly to concanavalin A–Sepharose (R. T. Fernley, J. P. Coghlan & R. D. Wright, unpublished work), as does the rat salivary enzyme [12]. The enzyme *N*-glycanase removes carbohydrate residues attached to asparagine residues of proteins [22]. Treatment of high-M_r carbonic anhydrase with this enzyme

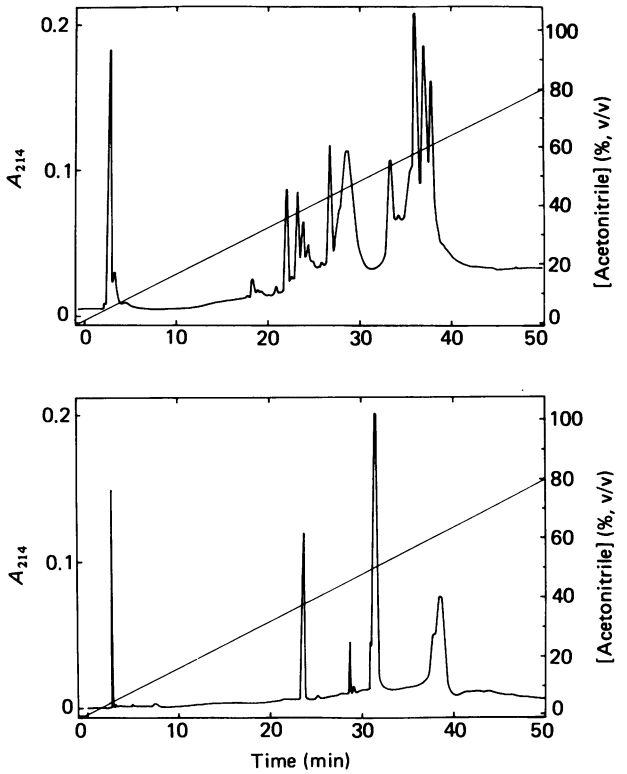

Fig. 8. Comparative CNBr-cleavage peptide mapping of sheep carbonic anhydrase

Samples (2 mg each) of reduced and carboxymethylated high-M_r carbonic anhydrase and of carbonic anhydrase II were digested with CNBr overnight, and the peptides were analysed by h.p.l.c. on a μBondapak C_{18} reversed-phase column (250 mm × 4.2 mm) with a linear acetonitrile gradient (5–100 %, v/v) in 0.1 % (v/v) trifluoroacetic acid. The column effluent was monitored at 214 nm. The upper panel shows the profile for the high-M_r carbonic anhydrase and the lower panel the profile for carbonic anhydrase II.

decreases its apparent subunit M_r to 36000. No evidence of lower-M_r bands could be found. The M_r of sheep carbonic anhydrase II treated with this enzyme was unchanged, indicating that no proteolytic activity was present in the *N*-glycanase preparation. Thus the high-M_r parotid enzyme appears to have a substantial carbohydrate content, in contrast with the carbonic anhydrase II enzyme, which has none and has no potential *N*-glycosylation sequences.

The high-M_r carbonic anhydrase zinc content was measured as 0.8 g-atom of zinc/mol of subunit (M_r 45000), indicating that there is one zinc atom/subunit. Like all other carbonic anhydrases, the enzyme is inhibited by azide and sulphonamides, implying it has a similar active site to other carbonic anhydrases. As reported previously [9], it is 2-fold less sensitive to acetazolamide inhibition and 10-fold less sensitive to benzolamide (CL 11366) inhibition than is carbonic anhydrase II. Membrane-bound [23,24] and the rat saliva [12] carbonic anhydrases are similarly less sensitive to sulphonamide inhibition than are the corresponding carbonic anhydrase II isoenzymes.

The CNBr-cleavage peptide maps of the two proteins are significantly different, indicating they are not closely related structurally. The amino acid composition of the high-M_r enzyme indicates that there are seven or eight methionine residues present, assuming there are about 320 amino acid residues/subunit, and nine main CNBr-cleavage peaks are observed. Carbonic anhydrase II has three methionine residues, and four peptides are observed (one as a shoulder of a larger peak). The lack of cross-reactivity between the antibodies and the antigens would confirm this view, as does the difference in *N*-termini. The relationship between this protein and other members of the carbonic anhydrase family remains to be fully established. However, this enzyme is found in saliva, is glycosylated and has a free *N*-terminus, in contrast with isoenzymes I, II and III, which are intracellular, not glycosylated and have *N*-termini that are *N*-acetylated. This supports the idea that it is a secreted enzyme, as has been suggested by Feldstein & Silverman [12]. Thus it would form a new class of carbonic anhydrase isoenzyme. Since Wistrand [7] has designated membrane-bound carbonic anhydrases as carbonic anhydrase IV, and the mitochondrial enzyme has been called carbonic anhydrase V [25], the secreted isoenzyme could be designated carbonic anhydrase VI.

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REFERENCES

1. Maren, T. H. (1967) Physiol. Rev. **47**, 595–781
2. Lamb, J. E. (1977) Life Sci. **20**, 393–406
3. Carter, M. J. (1972) Biol. Rev. Cambridge Philos. Soc. **47**, 465–513
4. Bundy, H. F. (1977) Comp. Biochem. Physiol. B57, 1–7
5. Carter, N., Jeffery, S., Shiels, A., Edwards, Y., Tipler, T. & Hopkinson, D. A. (1979) Biochem. Genet. **17**, 837–854
6. Tashian, R. E. (1977) Isozymes Curr. Top. Biol. Med. Res. **2**, 21–62
7. Wistrand, P. J. (1984) Ann. N.Y. Acad. Sci. **429**, 195–206
8. Whitney, P. L. & Briggle, T. V. (1982) J. Biol. Chem. **257**, 12056–12059
9. Fernley, R. T., Wright, R. D. & Coghlan, J. P. (1979) FEBS Lett. **105**, 299–302
10. Fernley, R. T., Congiu, M., Wright, R. D. & Coghlan, J. P. (1984) Ann. N.Y. Acad. Sci. **429**, 212–213
11. Feldstein, J. B. & Silverman, D. N. (1984) Ann. N.Y. Acad. Sci. **429**, 214–215
12. Feldstein, J. B. & Silverman, D. N. (1984) J. Biol. Chem. **259**, 5447–5453
13. Armstrong, J. McD., Myers, D. V., Verpoorte, J. A. & Edsall, J. T. (1966) J. Biol. Chem. **241**, 5137–5149
14. Andrews, P. (1965) Biochem. J. **96**, 595–606
15. Hammond, K. S. & Papermaster, D. S. (1976) Anal. Biochem. **74**, 292–297
16. Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tulbert, N. E. (1978) Anal. Biochem. **87**, 206–210
17. Angeletti, R. H., Bradshaw, R. A. & Wade, R. D. (1971) Biochemistry **10**, 463–469
18. Steers, E. Jr., Craven, G. R. & Anfinsen, C. B. (1965) J. Biol. Chem. **240**, 2478–2484
19. Tanis, R. J. & Tashian, R. E. (1971) Biochemistry **10**, 4852–4858

20. Moore, S. (1963) J. Biol. Chem. **238**, 235–237
21. Edelhoch, H. (1967) Biochemistry **6**, 1948–1954
22. Plummer, T. A., Jr., Elder, J. H., Alexander, S., Phelan, A. W. & Tarentino, A. L. (1984) J. Biol. Chem. **259**, 10700–10704
23. Wistrand, P. J. & Kinne, R. (1977) Pflügers Arch. **370**, 121–126
24. Maren, T. H. (1980) Ann. N.Y. Acad. Sci. **341**, 246–258
25. Venta, P. J., Montgomery, J. C. & Tashian, R. E. (1987) Isozymes Curr. Top. Biol. Med. Res. **14**, 59–72

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