BBA 66699

CARBONIC ANHYDRASE FROM *NEISSERIA SICCA*, STRAIN 6021
II. PROPERTIES OF THE PURIFIED ENZYME

J. BRUNDELL, S. O. FALKBRING AND P. O. NYMAN
Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Fack, S-402 20 Göteborg 5 (Sweden)

(Received April 19th, 1972)

SUMMARY

1. Physical and chemical properties of carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) purified from *Neisseria sicca*, strain 6021, have been investigated. Sedimentation equilibrium studies gave a molecular weight of 28 600. A molecular weight near 30 000 is also supported from the close similarity of gel filtration properties with human carbonic anhydrase B.

2. The enzyme contains 0.21% of Zn corresponding to 0.92 Zn^{2+} per molecular weight of 28 600. Determination of the amino acid composition shows that the contents of tyrosine, tryptophan, and proline are considerably lower than for the mammalian enzymes. The nitrogen content and the partial specific volume, estimated from the amino acid composition of the protein, are 17,7% and 0.731 ml/g, respectively.

3. Studies of the enzymatic properties show that the enzyme is a very efficient catalyst in the CO_2 hydration reaction. In activity it resembles the highly active forms (the C type) of the mammalian carbonic anhydrases. It has a weak esterase function towards *p*-nitrophenyl acetate. Both activities are inhibitable with carbonic anhydrase inhibitors of the sulfonamide type. The inhibitors tested seem to associate with the bacterial enzyme with binding strengths comparable in magnitude to those found for human erythrocyte carbonic anhydrases.

INTRODUCTION

The purification of a bacterial carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) from *Neisseria sicca*, strain 6021, was described in the preceding paper[1]. The present report is concerned with the properties of the purified enzyme and gives the results of determinations of molecular weight, amino acid composition, characterization of catalytic efficiency and inhibition by sulfonamides. The data obtained show that the bacterial enzyme in many respects resembles carbonic anhydrase from mammalian sources. It is a zinc-containing protein of about the same molecular size.

Abbreviation: SE-Sephadex, sulfoethyl Sephadex.

The specific enzyme activity in the CO_2 hydration reaction is comparable in magnitude to that of the highly active forms of mammalian erythrocyte carbonic anhydrases (*e.g.* human enzyme C). It is also strongly inhibited by aromatic and heterocyclic sulfonamides, from mammalian systems known as potent inhibitors of carbonic anhydrase[2]. These similarities would suggest that the bacterial carbonic anhydrase may be a valuable complement in the structure–function studies underway in several laboratories with the mammalian forms of the enzyme (recently reviewed in ref. 3).

MATERIALS AND METHODS

Preparation of carbonic anhydrases

The bacterial enzyme was purified as described in the preceding paper[1]. Human erythrocyte carbonic anhydrases B and C were obtained as described previously[4]. Human enzyme C from the final purification step was dialyzed against water and then used directly without any lyophilization (*cf.* ref. 5).

Enzyme activity and sulfonamide inhibition

Estimations of the activity of the bacterial enzyme as catalyst in the CO_2 hydration reaction and the inhibition of this activity were carried out as described in the preceding paper[1] using a Durrum–Gibson stopped-flow photometer. The buffer used was Veronal–H_2SO_4, pH 8.3, in a final concentration of 0.04 M. Comparative measurements were made in parallel with purified samples of human carbonic anhydrases B and C. Due to the differences between the various forms of the enzyme in ultraviolet absorption, specific enzyme activity has been expressed per mg of enzyme/ml instead of per absorbance unit ($A^{1\text{ cm}}_{280\text{ nm}}$).

Inhibition studies were carried out with three different sulfonamides: acetazolamide (Lederle, N.Y.), sulfanilamide (Eastman, N.Y.), and ethoxzolamide (UpJohn, Mich.). The sulfonamides were allowed to equilibrate with the enzyme–buffer mixture for at least 1 h before the measurements were carried out. K_i values were estimated under the assumption that the inhibition is non-competitive with respect to substrate[6] and were obtained as mean values from measurements at several different concentrations of inhibitors.

The esterase activity of the bacterial enzyme and its inhibition was studied with *p*-nitrophenyl acetate as substrate and acetazolamide as inhibitor. The experimental conditions were essentially the same as described elsewhere[7].

Amino acid analysis

Amino acid analyses were carried out with a Spinco Model 120 B amino acid analyzer. Treatment of samples was performed essentially as described by Moore and Stein[8]. The protein was hydrolyzed in 6 M HCl at 110 °C for 20 and 70 h. The hydrolysates were evaporated in a rotating evaporator, dissolved in 0.2 M sodium citrate buffer, pH 2.2, and analyzed. To correct for adventitious ammonia in the reagents a blank without protein was run parallel. Oxidation with performic acid was carried out as described by Moore[9]. After dilution with water the samples were lyophilized, hydrolyzed with 6 M HCl for 20 h and the cysteic acid content was determined by the amino acid analyzer.

The tryptophan content of the bacterial enzyme was determined by "Proce-

dure K" of Spies and Chambers[10] and by the method of Bencze and Schmid[11]. The concentration of enzyme was determined by amino acid analysis after acid hydrolysis of an aliquot of the starting solution. Human carbonic anhydrase B, which has a known content of tryptophan, was run parallel. The tyrosines of human enzyme B were found to be incompletely ionized in 0.1 M NaOH and the determination according to Bencze and Schmid was therefore carried out in the presence of 8 M urea as suggested by Wetlaufer[12]. A Cary 15 spectrophotometer equipped with 1-cm light-path cuvettes was used. During 30 min four spectra from 270 to 350 nm were recorded to obtain information about any destruction of tyrosine and tryptophan (*cf.* ref. 10) but no such effect was observed.

Zinc content

Determinations of zinc were carried out with a Perkin Elmer Model 303 atomic absorption spectrometer. The solutions of bacterial enzyme to be analyzed were dialyzed agianst deionized water for 3–5 days and the protein concentration was determined by measurement of the absorbance at 280 nm.

Ultraviolet absorption

The ultraviolet absorption spectrum of the bacterial enzyme was recorded with a Zeiss RPQ 20 A recording spectrophotometer. The specific absorbance at 280 nm was determined in the following way: The absorbance of the solution was measured and the nitrogen content was determined by micro-Kjeldahl analysis on duplicate samples. To avoid contamination by non-protein nitrogen, the final step in the purification of the enzyme (sulfoethyl Sephadex (SE-Sephadex) chromatography) had been carried out in sodium phosphate buffer (see ref. 1) and the enzyme had afterwards been dialyzed against deionized water. The value for the absorbance at 280 nm per mg N/ml obtained in this way was converted to absorbance per mg of protein/ml using the nitrogen content of the protein.

Partial specific volume

The partial specific volume of the bacterial enzyme was calculated from the amino acid composition by using the values for the specific volumes of the amino acid residues given by Cohn and Edsall[13]. The amount of ammonia registered in the amino acid analysis was regarded as representing amide ammonia and it was ascribed to asparagine and glutamine, respectively, in relation to the amounts of aspartic and glutamic acids.

Sedimentation studies

The experiments were carried out with a Spinco Model E ultracentrifuge at 59 780 rev./min and 20$\pm$ 0.1 °C. The enzyme solutions to be run were dialyzed against sodium phosphate buffer, pH 6.5, $I = 0.05$, containing 0.10 M NaCl. The sedimentation coefficients obtained were corrected to values corresponding to a water solution ($s_{20,w}$) according to Svedberg and Pedersen[14].

Molecular weight

The molecular weight of the bacterial carbonic anhydrase was estimated by the sedimentation equilibrium technique, as described by Yphantis[15]. Using a stan-

dard double-sector cell and Rayleigh interference optics, the ultracentrifuge was run at a constant speed of 39 460 rev./min at 25 ± 0.1 °C for 20 h. The initial protein concentration was 0.04% and the enzyme had been dialyzed for two days against sodium phosphate buffer, pH 6.5, $I = 0.05$, containing 0.10 M NaCl. To correct for optical distortion in the cell, a second run was performed under the same conditions but with buffer only. The details in the experimental procedures and elucidation of the results were similar to those given by Butzow[16]. The weight-average molecular weight was calculated[15], utilizing the values for the partial specific volume of the bacterial enzyme (0.731 ml/mg) estimated from the amino acid composition.

The molecular size of the bacterial carbonic anhydrase was also investigated by gel filtration. A preparation of bacterial enzyme purified by DEAE-chromatography (*Step 2* in the purification procedure described in the preceding paper[1]) was chromatographed on a column packed with polyacrylamide gel (Bio-Gel P-30, Biorad Laboratories, Calif.) which had previously been calibrated with a sample of purified human carbonic anhydrase B. Experimental conditions are described in the legend to Fig. 2.

Circular dichroism measurements

Circular dichroism measurements were carried out at room temperature (about 22 °C) using a Cary Model 60 recording spectropolarimeter, equipped with a Cary 6002 circular dichroism attachment. Measurements in the region 250 to 300 nm were made in 1- and 5-cm cuvettes, in the region 225 to 250 nm in a 1-mm cuvette and in the region 195 to 225 nm in a 0.1-mm cuvette. The enzyme had been dialyzed for 2 days against 0.05 M sodium phosphate buffer, pH 7.0. Measured ellipticities, θ, were converted to mean residue ellipticities, $[\theta]$, according to the formula $[\theta] = (\theta \cdot W_M)/100\ dc$, where W_M is the mean residue weight, d is the path length in decimeters, and c the protein concentration in g/cm^3.

RESULTS

Molecular weight and sedimentation

The molecular weight determination of the bacterial enzyme by sedimentation equilibrium is illustrated in Fig. 1. The slope of the line, $2.60 \cdot 10^{-2}$ mm^{-2}, is proportional to half of the effective reduced molecular weight. The weight average of the molecular weight was calculated from this value according to Yphantis[15]. The value obtained, 28 600, is consistent with the gel filtration result shown in Fig. 2. In this experiment the bacterial enzyme emerges from the column after an elution volume of 57 ml which is exactly the same as obtained for human carbonic anhydrase B, which, on the basis of extensive studies in various laboratories[6,17,18], has been assigned a molecular weight near 30 000.

Determinations of the sedimentation coefficient for the bacterial carbonic anhydrase gave values of 2.37 and 2.42 S at initial protein concentrations of 2.9 and 0.93 mg/ml, respectively. A run of human carbonic anhydrase B under the same conditions carried out in parallel gave 2.68 S at an enzyme concentration of 3.3 mg/ml which is considerably lower than most of the values reported for this protein in the literature (*cf.* ref. 6).

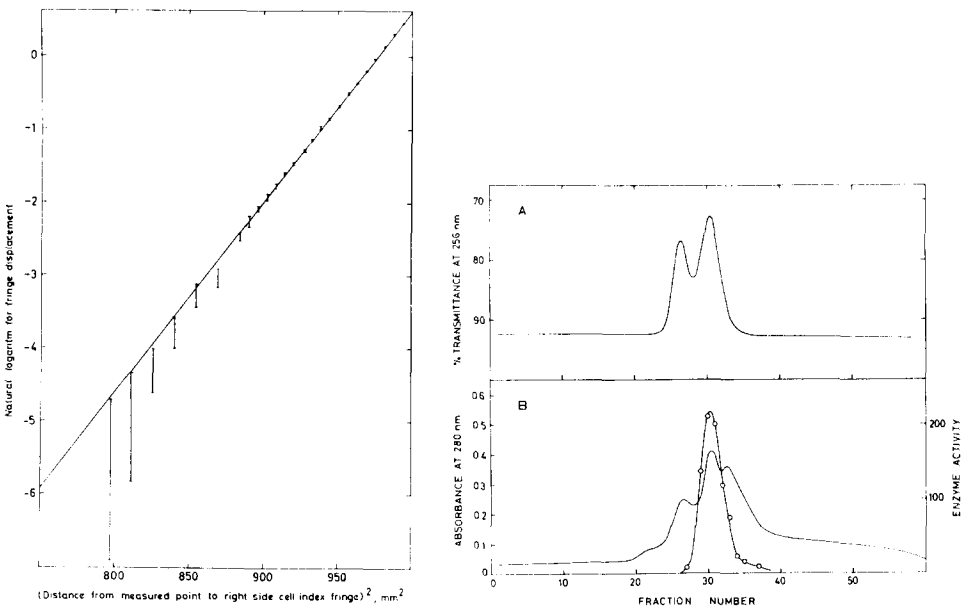

Fig. 1. Determination of the molecular weight of bacterial carbonic anhydrase by equilibrium ultracentrifugation at 39 460 rev./min. Temperature: 25 ± 0.1 °C. Duration: 20 h. The natural logarithm for the blank-corrected fringe displacements ($z - z_0$) has been plotted against the square of the position variable, *i.e.* the distance between a measured point (R) and the right side cell index fringe (R_I). The slope of the line equals $\sigma_w/2$ where σ_w is a function of the position and concentration and is directly related to the effective reduced molecular weight and the weight average of the molecular weight (*cf.* ref. 15).

Fig. 2. Comparison of gel-filtration properties of bacterial carbonic anhydrase and human carbonic anhydrase B. The proteins were chromatographed by successive runs on the same column. The column (1.2 cm × 117 cm) was packed with polyacrylamide gel (Bio-Gel P-30) and equilibrated with Tris–HCl buffer, pH 7.5. Lyophilized protein samples were dissolved in this buffer and in each run 1 ml of protein solution was applied to the column. The chromatograms were developed at a flow rate of 6.3 ml/h which was held constant by a peristaltic pump. Fraction volume: 1.9 ml. Temperature: 8–10 °C. A. Purified human carbonic anhydrase B. The diagram is a replotting of an automatic recording of the chromatography with a Uvicord (LKB, Stockholm, Sweden). The two peaks are ascribed to dimers and monomers of the protein, respectively. B. Partially purified preparation of carbonic anhydrase from *N. sicca*. ———, absorbance at 280 nm (1-cm cell); ○—○, enzyme activity. Recovery of enzyme activity was estimated to 97%.

Amino acid composition

The results of the amino acid analyses of bacterial carbonic anhydrase are shown in Table I. For comparison, the compositions of some other carbonic anhydrases are also given. The data for the bacterial enzyme have been treated under the assumption that the protein has a molecular weight of 28 600, as determined by the ultracentrifuge, and, with the exception for the zinc ion, is built up entirely by amino acid residues. An estimation of the molecular weight from the amino acid composition assuming that the various amino acids should occur in or close to integral numbers of residues[17] gives some indication for a molecular weight of about 24 000 for the protein part of the enzyme molecule. The discrepancy compared to the value determined with the ultracentrifuge would perhaps suggest that the enzyme molecule contains constituents which have not yet been detected. Analyses for phosphate, carbohydrates

TABLE I

AMINO ACID COMPOSITION OF BACTERIAL CARBONIC ANHYDRASE

The figures given are the mean values from analyses of four different preparations of the enzyme. The deviations are the average values of the absolute deviations from the mean. The compositions of human erythrocyte carbonic anhydrases[3] and of the parsley enzyme[20] are given for comparison.

Amino acid	*Bacterial enzyme*			*Human enzyme C*	*Human enzyme B*	*Parsley enzyme*
	Residues in moles per 28 600 g of protein	*Nitrogen (g) per 100 g of protein*	*Nearest integral value*	*Residues in moles per 30 000 g of protein*		*Residues in moles per 28 150 g of protein*
Lysine	25.4 ± 0.3	2.49	25	24	18	21
Histidine	11.7 ± 0.4	1.71	12	12	11	5
Amide ammonia	35.4 ± 1.4[a,b]	1.73	35	21	26	—
Arginine	4.9 ± 0.3	0.97	5	7	7	5
Tryptophan	3.6 ± 0.3[c]	0.36	4	7	6	3
Aspartic acid	28.8 ± 0.6	1.41	29	29	31	24
Threonine	20.3 ± 0.5[a]	0.99	20	13	14	10
Serine	15.4 ± 1.1[a]	0.75	15	19	30	18
Glutamic acid	28.9 ± 0.8	1.41	29	25	22	21
Proline	13.4 ± 0.4	0.66	13	17	18	22
Glycine	18.3 ± 0.6	0.89	18	22	16	22
Alanine	25.6 ± 0.2	1.25	26	13	19	17
Valine	17.8 ± 0.1[d]	0.87	18	17	17	24
Methionine	2.4 ± 0.2	0.12	2	1	2	4
Isoleucine	6.4 ± 0.2[d]	0.31	6	9	10	8
Leucine	17.1 ± 0.5	0.84	17	25	20	21
Tyrosine	5.7 ± 0.2	0.28	6	8	8	8
Phenylalanine	12.1 ± 0.1	0.59	12	12	11	17
Half-cystine	2.2 ± 0.2[e]	0.11	2	1	1	7
Total	260.0 ± 6.9	17.74	259	261	261	257

[a] Values obtained by extrapolating to zero time of hydrolysis according to Moore and Stein[8].

[b] Omitted from the summation.

[c] Average of two determinations according to Spies and Chambers[10] and Bencze and Schmid[11].

[d] Values from the 70 h hydrolysis only.

[e] Determined on hydrolysates of performic acid-oxidized samples according to Moore[9].

etc. have not yet been carried out. The amino acid analyses give evidence, however, for the absence of aminohexoses in the bacterial carbonic anhydrase.

Nitrogen and zinc contents

The nitrogen content calculated from the amino acid composition amounts to 17.7%. Corresponding figures similarly estimated from the compositions of human and bovine carbonic anhydrases are shown in Table II.

The zinc content of the bacterial carbonic anhydrase was determined to be 0.21%. This value which is very close to those of various mammalian carbonic anhydrases (see Table II) corresponds to 0.92 zinc ions per protein molecule with a particle weight of 28 600.

Ultraviolet absorption

The ultraviolet absorption of the bacterial carbonic anhydrase is typical for a

TABLE II

PROPERTIES OF BACTERIAL CARBONIC ANHYDRASE COMPARED WITH DATA FOR MAMMALIAN AND PLANT CARBONIC ANHYDRASES

The nitrogen content for the bacterial enzyme was calculated from the amino acid composition in Table I and used in the estimations of specific absorbance and zinc content as described in Materials and Methods. The values within parentheses are the data for mammalian enzymes reported earlier[17] based on determinations of nitrogen on trichloroacetic acid precipitated protein samples. The values for the mammalian enzymes given without parentheses are the nitrogen contents estimated from the amino acid compositions of these proteins[17] and specific absorbances and zinc contents re-evaluated by the utilization of the nitrogen contents derived in this way. The data for the parsley enzyme are from Tobin[20].

	Zinc content (%)	*Nitrogen content (%)*	*Absorbance at 280 nm (1-cm cell) of a 0.100% solution of protein*	*Partial specific volume (ml/g)*
Human enzyme B	0.21 (0.21)	17.2 (16.9)	1.66 (1.63)	0.729
Human enzyme C	0.21 (0.21)	17.1 (16.9)	1.89 (1.87)	0.735
Bovine enzyme	0.22 (0.22)	17.3 (17.0)	1.93 (1.90)	0.735
Parsley enzyme	0.22	—	1.10	0.736
Bacterial enzyme	0.21	17.7	1.25	0.731

simple protein. It shows a maximum at 280 nm and a minimum at 251 nm. The specific absorbance at 280 nm was taken as the absorbance in a 1-cm cell of a solution of which the nitrogen content had been determined by micro-Kjeldahl analysis. The average value obtained from two such determinations was 7.02 absorbance units (1-cm cell)/mg N per ml. Using a nitrogen content of 17.7%, estimated from the amino acid composition, the absorbance of a 0.10% solution of bacterial carbonic anhydrase was estimated to be 1.25 in a 1-cm cell. With a molecular weight of 28 600 this figure corresponds to a molar absorbance coefficient of 35 750 $M^{-1} \cdot cm^{-1}$. The bacterial carbonic anhydrase thus has an ultraviolet absorption which is considerably lower than the values reported for mammalian carbonic anhydrases shown for comparison in Table II. This finding is in accordance with the lower contents of aromatic amino acids, notably tyrosine and tryptophan.

Sulfonamide inhibition

The results of the activity and inhibition studies are given in Table III. It is evident that the efficiency of the bacterial carbonic anhydrase as catalyst in the hydration of CO_2 is comparable in magnitude to that of human erythrocyte carbonic anhydrases which are known as very efficient catalysts. As in the case of mammalian carbonic anhydrases, aromatic and heterocyclic sulfonamides act as strong inhibitors to the bacterial enzyme. Besides the three sulfonamides quoted in Table III, the inhibitory effect on the bacterial carbonic anhydrase has also been found for other sulfonamides such as chlorothiazide, dichlorophenamide, *p*-iodobenzenesulfonamide, and *p*-toluenesulfonamide.

Esterase activity

Preliminary determinations with *p*-nitrophenyl acetate as substrate show that the bacterial enzyme has an esterase activity. This activity is very weak (under the conditions used here about 10% of that of human carbonic anhydrase C) and rather

TABLE III

COMPARISON OF BACTERIAL CARBONIC ANHYDRASE WITH HUMAN ERYTHROCYTE CARBONIC ANHYDRASES IN SPECIFIC ENZYME ACTIVITY AND SULFONAMIDE INHIBITION

The data refer to the CO_2 hydration reaction which has been measured as described in preceding paper[1]. Dissociation constants (K_i) for the enzyme–inhibitor complexes have been estimated assuming non-competitive inhibition. The final concentration of the enzyme in the assay mixture was $0.8 \cdot 10^{-8}$–$4.6 \cdot 10^{-8}$ M. The sample of acetazolamide used in the experiment contained 1.5 equiv. of sodium and 1 equiv. of water[7] which were corrected for.

	Specific enzyme activity (relative values)	K_i *(M)*		
		Acetazolamide	*Sulfanilamide*	*Ethoxzolamide*
Human enzyme B	1	$50 \cdot 10^{-8}$	$20 \cdot 10^{-6}$	$\approx 45 \cdot 10^{-9}$
Human enzyme C	6.6	$2 \cdot 10^{-8}$	$5 \cdot 10^{-6}$	$\approx 4 \cdot 10^{-9}$
Bacterial enzyme	5.8	$5 \cdot 10^{-8}$	$3 \cdot 10^{-6}$	$10 \cdot 10^{-9}$

high amounts of enzyme would be required for a careful characterization of this reaction. That the esterase activity is an inherent property of the active site of bacterial carbonic anhydrase is suggested by the finding that the major part of this activity, but not all (*cf.* footnote 7 in ref. 19), can be abolished by the addition of acetazolamide.

Circular dichroism

The ultraviolet circular dichroism spectrum of bacterial carbonic anhydrase, shown in Fig. 3, has positive bands in the region 250 to 300 nm and below about 200 nm. The number of bands associated with near ultraviolet transitions of aromatic amino acids is difficult to determine from the spectrum and no definite assigne-

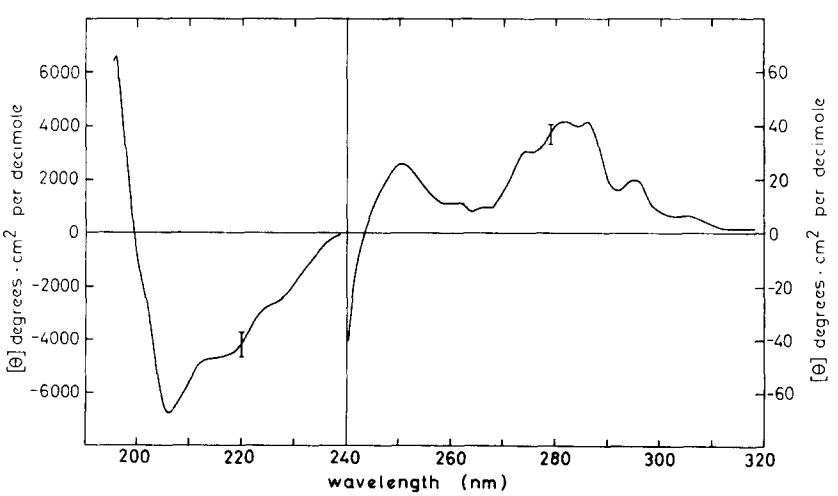

Fig. 3. Circular dichroism of bacterial carbonic anhydrase in the ultraviolet region. Measurements were made at room temperature (about 22 °C) on a Cary Model 60 recording spectropolarimeter equipped with a Cary 6002 circular dichroism attachment. The mean residue ellipticity, $[\theta]$, was calculated as described under Materials and Methods. The enzyme had been dialyzed for 2 days against 0.05 M sodium phosphate buffer, pH 7.0.

ment of bands could be made to various kinds of residues. There are strong negative bands in the region 200 to about 240 nm with the deepest extreme at about 207 nm.

DISCUSSION

Carbonic anhydrase has previously been characterized from animal as well as from plant material. The forms from mammalian erythrocytes, in particular the human and bovine forms, have been thoroughly investigated[3]. The carbonic anhydrase dealt with in the present study is of a distant origin, namely a bacterial species. It was taken up for study as a form of the enzyme with which it may be possible to use genetic methods as an additional tool for studies on the structure–function relationship of the enzyme (see Discussion in the preceding paper[1]).

The results of the present study can be utilized for a comparison of the bacterial carbonic anhydrase with earlier characterized forms of the enzyme. The mammalian forms are monomeric proteins with molecular weights around 30 000[3]. The enzyme isolated from plants has a higher molecular weight; 180 000 has been reported for the parsley enzyme[20], 194 000 for the pea enzyme[21], and 140 000–148 000 for the carbonic anhydrase from spinach[22]. Evidence has been obtained suggesting that the plant enzymes consist of polymeric arrangements of subunits; in the case of the parsley and pea enzymes the subunit molecular weight has been reported to be 29 000–30 000. According to a preliminary report[23], the polymeric nature may be a characteristic of carbonic anhydrase from dicotyledonic plants while the enzyme from monocotyledones appears to occur in a non-aggregated state. Non-mammalian animal carbonic anhydrase purified from two different species of shark[24] were found to have molecular weights in the range of 36 000 to 40 000 while a value of 28 000 has been reported for the avian enzyme from chicken[25]. The figure for the bacterial enzyme in the present investigation, obtained by sedimentation equilibrium and gel filtration, is near or slightly below 30 000. This means that the bacterial enzyme behaves as a monomeric protein in contrast to the carbonic anhydrases from parsley, pea, and spinach and in size the monomer resembles the mammalian and avian forms of the enzymes.

The data in Table II show that the bacterial carbonic anhydrase is a zinc-containing protein. The zinc content corresponds to one mole per approx. 30 000 g of protein. This is in agreement with other carbonic anhydrases[3] which have been shown to contain 1 zinc ion per protein molecule or, as in the case of the parsley and pea enzymes, per subunit.

A notable feature in the amino acid composition of the bacterial carbonic anhydrase (Table I) is the low content of sulfur-containing amino acids. The figure for half-cystine corresponds to about two residues per enzyme molecule. Theoretically, that would allow for the presence of a disulfide bridge in the molecule. Whether the half-cystines occur in that form or as cysteine residues, has not yet been investigated. A low content of sulfur-containing amino acids has previously been shown to be a characteristic feature of the compositions of the mammalian carbonic anhydrases[3]. The enzyme forms from ox and pig erythrocytes are devoid of half-cystine while other mammalian forms investigated contain a single residue or, as in the case of horse carbonic anhydrase B, two residues. The plant enzymes from parsley[20], pea[21] and spinach[22], on the other hand, as well as the elasmobranch carbonic anhy-

drase from sharks[24] and the avian carbonic anhydrase from chicken[25], are rich in half-cystines. In the shark enzymes they have been shown to occur largely in the form of disulfide bridges.

For the conformation of the bacterial protein it is interesting to note that the content of proline is lower than the values found in carbonic anhydrases analyzed previously. Furthermore, the bacterial enzyme has a lower content of tyrosine and tryptophan than, for example, the mammalian forms while the number of phenylalanine residues appears to be about the same. This is notable, since in the X-ray structure of human carbonic anhydrase C[26,3] clusters of aromatic amino acid residues have been recognized which may play a role in the stability of the native conformation as indicated by the finding that the residues involved show up as conservative elements in the amino acid sequences of mammalian carbonic anhydrases[27].

The circular dichroism spectrum of the bacterial carbonic anhydrase (Fig. 3) shows, in similarity with animal carbonic anhydrases, a multiplicity of bands in the ultraviolet region. In the near ultraviolet range the bacterial enzyme has a positive ellipticity where the animal carbonic anhydrases show a negative one[24,28,29]. Similar positive bands may exist for the animal enzymes but in that case they are superimposed by a strong negative band centered around 270 nm. This negative band which has been ascribed to tyrosine side chains[28] does not seem to be present in the spectrum of the bacterial enzyme, at least not to the same degree. Part of the complexity in the near ultraviolet dichroism spectra of the mammalian enzymes has been attributed to tryptophan transitions[30]. In view of the dissimilarities between the bacterial and animal carbonic anhydrases in their contents of tyrosine and tryptophan residues, differences in the near ultraviolet region of their circular dichroism spectra do not appear as surprising, but more specific structural differences may also be involved.

The extreme at 207 nm would, by comparison with the model spectra of Greenfield and Fasman[31], indicate the presence of an α-helix in the bacterial carbonic anhydrase. The magnitude of the band would suggest a helical content of about 10%. This figure is lower than for human carbonic anhydrase C where X-ray diffraction studies to 2 Å resolution[26,3] show a helical content of about 20% and β-structure, mainly antiparallel, to about 37% of the polypeptide chain. The circular dichroism of the two proteins in the far ultraviolet region, where secondary structures would be expected to influence, are not very similar. In the general shape of the spectrum the bacterial enzyme appears to show a greater resemblance to several other proteins such as lysozyme, ribonuclease, and carboxypeptidase A. Compared to the mammalian carbonic anhydrases these proteins have less complex circular dichroism spectra[31]. Since they have been shown to contain appreciable amounts of α-helix as well as β-structure both these elements of secondary structure may also occur in the bacterial carbonic anhydrase.

The mammalian carbonic anhydrase in general consists of a mixture of two types of enzyme, in the literature often referred to as forms B and C[3]. They differ considerably from each other in amino acid sequence and in the functioning of their active sites as reflected in dissimilarities in catalytic properties[32] and inhibitor binding[33]. The C type carbonic anhydrase is a more efficient catalyst in the CO_2 hydration reaction than the B enzyme. The difference in specific enzyme activity obtained with the assay method used here is illustrated in Table III where the two forms of

the human carbonic anhydrase are compared to the bacterial enzyme. The specific enzyme activity for the bacterial carbonic anhydrase is comparable in magnitude to that of human enzyme C. Also in sulfonamide inhibition the bacterial protein appears to resemble human enzyme C more closely than the B form (see Table III). The plant enzyme from parsley is known to be a less efficient catalyst in the CO_2 hydration reaction than the mammalian C forms[20]. Whether the plant carbonic anhydrases are inhibitable with sulfonamides has not yet been settled conclusively. Among reports available the more recent ones[21,34,35] would suggest that inhibition really occurs but perhaps more weakly than for the mammalian and bacterial enzymes.

Table IV shows a comparison in amino acid composition of the bacterial enzyme with carbonic anhydrases of various origins. The figures in the table represent the minimum number of amino acid residues that must be replaced in order to derive the various forms of carbonic anhydrase from the bacterial enzyme. Without exception, the figures obtained are lower for the C enzyme than for the B enzyme from the same

TABLE IV

COMPARISON OF THE AMINO ACID COMPOSITION OF BACTERIAL CARBONIC ANHYDRASE WITH THOSE OF OTHER CARBONIC ANHYDRASES

The table shows the minimum number of amino acid residues which must be replaced in order to derive the composition of various carbonic anhydrases from the composition of the bacterial enzyme. The amino acid compositions have been obtained from the references quoted in the last column. Data for cystine and tryptophan were not always available and one of the columns shows the results obtained with these residues omitted. For species showing the polymorphism of having both B and C type carbonic anhydrase, both forms have been included.

Bacterial carbonic anhydrase compared to:	*Minimum number of variant residues*		*Ref. No.*
	Half-cystine and tryptophan omitted	*Half-cystine and tryptophan included*	
Horse C	53	55	3
Guinea pig C	53	57	3
Man C	54	58	3
Deer mouse C (*Peromyscus maniculatus*)	55	—	34
Green monkey C (*Cercopithecus aethiops*)	57	—	34
Rhesus monkey C (*Macaca mulatta*)	58	62	3
Ox[a]	59	64	3
Pig C	60	64	3
Pig-tailed macaque C (*Macaca nemestrina*)	61	—	34
Deer mouse B	61	—	34
Chicken[a]	62	70	25
Man B	65	68	3
Green monkey B	68	—	34
Horse B	69	70	3
Guinea pig B	70	73	3
Pig-tailed macaque B	71	—	34
Rhesus monkey B	79	83	3
Pig B	80	85	3
Parsley	80	86	3

[a] Bovine erythrocyte carbonic anhydrase has often been denoted B. However, for good reasons, discussed elsewhere[3], it would be better regarded as a type C carbonic anhydrase as would also the single enzyme form found in chicken erythrocytes[25].

species. Furthermore, all enzymes of the C type with the exception of the chicken carbonic anhydrase become more closely ranked to the bacterial enzyme than any of the B forms. This finding would indicate that the bacterial carbonic anhydrase may be more similar in structure to the C type than to the B type of the mammalian carbonic anhydrase.

The finding that the bacterial enzyme, in properties of the active site (Table III) as well as in amino acid composition (Table IV), comes closer to the C type of the mammalian carbonic anhydrase than to the B type can be explained in various ways. The mammalian B and C forms have, on the basis of similarities in amino acid sequence, been regarded as arising from a common evolutionary origin and are suggested to have evolved by a gene duplication in the evolutionary past[3]. From certain evidence[36] it has been proposed that the C type would, from an evolutionary point of view, be an older type of carbonic anhydrase than the B form which may be a protein which has undergone, and perhaps is still undergoing, a faster evolutionary change. In this way enzyme B may have become considerably differentiated from the structure present when the structural gene for carbonic anhydrase became duplicated yielding the polymorphism we can nowadays recognize in mammalian systems. The change in the C enzyme would have been comparatively smaller, presumably due to a stronger selective pressure against mutational variants. The data in Tables III and IV would seem to provide additional support for such an idea. The reasoning assumes, however, that the bacterial enzyme represents an early form of carbonic anhydrase in evolution. It should be kept in mind that we do not know how widespread the enzyme may be among bacteria. So far, it has clearly and repeatedly been demonstrated only for species within the genus *Neisseria* while reports[37,38] about its occurrence in a few other bacteria remain to be confirmed. *Neisseria* species occur as parasites in mammalian systems and many strains of the genus show an ability to undergo genetic transformation, even interspecific, by DNA present in the surroundings[39,40].

ACKNOWLEDGEMENTS

The authors wish to thank Professor B. G. Malmström for continued interest and support, Dr J. Butzow for advice and help with the sedimentation equilibrium centrifugation, Mrs K. Årén, Department of Analytical Chemistry, Göteborg, for help with the zinc analyses, and Mr L. Strid and Mrs B. Liepins for help with the amino acid analyses. We also wish to thank the Wallenberg foundation for generous support that made the circular dichroism studies possible, and UpJohn Co., Michigan, for a generous gift of ethoxzolamide. The investigation was supported by research grants from the Swedish Medical and Natural Science Research Councils, the Swedish Board for Technical Development, and the Institute of General Medical Sciences, U. S. Public Health Service (GM 12 280–06).

REFERENCES

1 L. Adler, J. Brundell, S. O. Falkbring and P. O. Nyman, *Biochim. Biophys. Acta*, 284 (1972) 298.
2 T. H. Maren, *Physiol. Rev.*, 47 (1967) 595.
3 S. Lindskog, L. E. Henderson, K. K. Kannan, A. Liljas, P. O. Nyman and B. Strandberg,

in P. D. Boyer, *The Enzymes*, Vol. 5, Academic Press, New York and London, 3rd ed., 1971, p. 587.
4 P. O. Nyman, *Biochim. Biophys. Acta*, 52 (1961) 1.
5 P. O. Nyman, *Abstr. Gothenburg Diss. Sci.*, 15 (1969).
6 J. M. Armstrong, D. V. Myers, J. A. Verpoorte and J. T. Edsall, *J. Biol. Chem.*, 241 (1966) 5137.
7 P. L. Whitney, G. Fölsch, P. O. Nyman and B. G. Malmström, *J. Biol. Chem.*, 242 (1967) 4206.
8 S. Moore and W. Stein, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 6, Academic Press, New York, 1963, p. 819.
9 S. Moore, *J. Biol. Chem.*, 238 (1963) 235.
10 J. R. Spies and D. C. Chambers, *Anal. Chem.*, 21 (1949) 1249.
11 W. L. Bencze and K. Schmid, *Anal. Chem.*, 29 (1957) 1193.
12 D. B. Wetlaufer, in C. B. Anfinsen, Jr, M. L. Anson, K. Bailey and J. T. Edsall, *Advances in Protein Chemistry*, Vol. 17, Academic Press, New York, 1962, p. 303.
13 E. J. Cohn and J. T. Edsall, *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, Reinhold, New York, 1943.
14 T. Svedberg and K. O. Pedersen, *The Ultracentrifuge*, Oxford University Press, London, 1940.
15 D. A. Yphantis, *Biochemistry*, 3 (1964) 297.
16 J. J. Butzow, *Biochim. Biophys. Acta*, 168 (1968) 490.
17 P. O. Nyman and S. Lindskog, *Biochim. Biophys. Acta*, 85 (1964) 141.
18 J. Reynaud, P. Santini, M. Bouthier, J. Savary and Y. Derrien, *Biochim. Biophys. Acta*, 171 (1969) 363.
19 P. L. Whitney, P. O. Nyman and B. G. Malmström, *J. Biol. Chem.*, 242 (1967) 4212.
20 A. J. Tobin, *J. Biol. Chem.*, 245 (1970) 2656.
21 W. Kisiel and G. Graf, *Phytochemistry*, 11 (1972) 113.
22 C. Rossi, A. Chersi and M. Cortivo, in R. E. Forster, J. T. Edsall, A. B. Otis and F. J. W. Roughton, *CO_2: Chemical, Biochemical and Physiological Aspects*, NASA, SP-188, Washington 1969, p. 131.
23 B. D. Patterson, C. A. Atkins and D. Graham, *Biochem. J.*, 125 (1971) 34 P.
24 J. R. Maynard and J. E. Coleman, *J. Biol. Chem.*, 246 (1971) 4455.
25 R. S. Bernstein and R. Schraer, *J. Biol. Chem.*, 247 (1972) 1306.
26 A. Liljas, K. K. Kannan, P. C. Bergstén, I. Waara, K. Fridborg, B. Strandberg, U. Carlbom, L. Järup, S. Lövgren and M. Petef, *Nature New Biol.*, 235 (1972) 131.
27 R. E. Tashian, R. J. Tanis and R. E. Ferrell, in M. Rørth and P. Astrup, *Oxygen Affinity of Hemoglobin and Red Cell Acid–Base Status, Alfred Benzon Symposium IV*, Munksgaard, Copenhagen, and Academic Press, New York, 1972, p. 353.
28 S. Beychok, J. McD. Armstrong, C. Lindblow and J. T. Edsall, *J. Biol. Chem.*, 241 (1966) 5150.
29 J. E. Coleman, in R. E. Forster, J. T. Edsall, A. B. Otis and F. J. W. Roughton, *CO_2: Chemical, Biochemical and Physiological Aspects*, NASA, SP-188. Washington, D.C., 1969, p. 141.
30 S. N. Timasheff, in P. D. Boyer, *The Enzymes*, Vol. 2, Academic Press, New York and London, 3rd, ed., 1970, p. 371.
31 N. Greenfield and G. D. Fasman, *Biochemistry*, 8 (1969) 4108.
32 R. G. Khalifah, *J. Biol. Chem.*, 246 (1971) 2561.
33 P. W. Taylor, R. W. King and A. S. V. Burgen, *Biochemistry*, 9 (1970) 2638.
34 R. G. Everson, *Phytochemistry*, 9 (1970) 25.
35 M. Ikemori and K. Nishida, *Physiol. Plant.*, 21 (1968) 292.
36 R. E. Tashian, D. C. Shreffler and T. B. Shows, *Ann. N.Y. Acad. Sci.*, 151 (1968) 64.
37 F. P. Veitch and L. C. Blankenship, *Nature*, 197 (1963) 76.
38 W. T. Shoaf and M. E. Jones, *Arch. Biochem. Biophys.*, 139 (1970) 130.
39 B. W. Catlin and J. S. Cunningham, *J. Gen. Microbiol.*, 26 (1961) 303.
40 K. Bövre, *Acta Pathol. Microbiol. Scand.*, 69 (1967) 123.