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PURIFICATION AND SOME PROPERTIES OF ERYTHROCYTE CARBONIC ANHYDRASE FROM THE EUROPEAN MOOSE

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SUMMARY

1. Carbonic anhydrase has been purified from erythrocytes of the European moose. Two forms (relative abundance, 4:1) with high specific activities (C-type) were separated by DEAE-cellulose chromatography. The moose appears to lack a low-activity (B-type) carbonic anhydrase in the blood which is similar to other ruminants.

2. The purified major and minor forms appeared homogeneous judging from polyacrylamide gel electrophoresis, ultracentrifugation and gel filtration experiments. The two forms have practically identical amino acid compositions. Each of them is composed of about 260 amino acid residues and one Zn^{2+} corresponding to a molecular weight of approx. 30 000.

3. The physical and chemical properties of the major form of moose carbonic anhydrase are very similar to the corresponding properties of the bovine or sheep enzymes. The amino acid sequence of a section of the peptide chain, presumably comprising two of the metal-liganding histidyl residues, is –Leu–Val–Glx–Phe–His–Phe–His–Trp–Gly–, which is identical to the corresponding sequences in the bovine and sheep enzymes. The C-terminal sequence, –Leu–Arg, exhibits an inhomology in the penultimate position compared to other ruminant carbonic anhydrases, which could be explained by a single point mutation.

INTRODUCTION

Most mammalian carbonic anhydrases (carbonate hydro-lyase, EC 4.2.1.1) exhibit a polymorphism mainly resulting from the presence of two isoenzymes; a high-activity and a low-activity form (designated C and B, respectively) with distinct amino acid compositions and sequences[1]. Additional polymorphism has also been observed in several species due to the existence of genetic variants or to the presence of low levels of secondary modifications of the two major isoenzymes[1].

The erythrocytes of ox[2], sheep[3] and goat[4] contain only high-activity forms of carbonic anhydrase. To investigate whether the lack of a low-activity erythrocyte

enzyme is restricted to species of the *Bovidae* family or if it is a general feature among the ruminants, we have isolated the enzyme from red cells of the European moose (*Alces alces alces*) belonging to the *Cervidae* (deer) family. During the course of our study Ashworth *et al.*[4] briefly reported that an unspecified deer species had only C-type red cell carbonic anhydrase.

MATERIALS AND METHODS

Initial purification

The starting material was citrated whole moose blood collected from several animals. Crude carbonic anhydrase was prepared using a modification of Method II of Keilin and Mann[5]. The erythrocytes were separated from plasma and leucocytes by centrifugatoin for 20 min at 2300 × *g* in an International serum centrifuge. The cells were then washed 3 times with an equal volume of cold isotonic NaCl solution (0.9%). Hemolysis was achieved by addition of half the volume of distilled water to the packed red cells. Hemoglobin was removed in the cold room (4–6 °C) by selective denaturation with a chloroform–ethanol mixture. To 1 l of hemolysate 800 ml 40% (v/v) ethanol–water and 400 ml chloroform were added under vigorous stirring for 5 min. The mixture was allowed to stand for 20 min before centrifugation (20 min, 1800 × *g*). The slightly yellow supernatant solution containing the enzyme was filtered through glass wool to remove particles. The enzyme extract was dialyzed against several changes of distilled water for 3 days and then concentrated to approx. 20% of the original volume by pressure dialysis in a Diaflo apparatus (PM-10 filter).

Ion-exchange chromatography

The subsequent purification was made similarly as described by Henderson and Henriksson[6] at 4 °C on a DEAE-cellulose column (2.8 cm × 45 cm; Whatman DE-23, W. and R. Balston, Kent, England) equilibrated with 0.01 M Tris–HCl buffer (pH 8.7). The pH of the concentrated protein solution was adjusted to 8.7 by addition of solid Tris. If necessary the solution was diluted so that the conductivity did not exceed that of the starting buffer. After application of the protein solution the column was washed with about 150 ml of starting buffer. Elution was performed step-wise with 0.04 and 0.15 M Tris–HCl buffer. Finally the column was washed with 0.5 M buffer. All pH values refer to measurements performed at room temperature.

Enzyme assays

The CO_2 hydration activity was assayed by a colorimetric method according to the procedure of Rickli *et al.*[7], and the definition of activity units used by these authors was employed; (activity units) = $10 \times (t_b - t_c)/t_c$, where t_b and t_c are the times for obtaining the color change of the indicator in the nonenzymic and enzyme-catalyzed reactions, respectively. The esterase activity was determined with *p*-nitrophenyl acetate as substrate according to Thorslund and Lindskog[8].

Isoelectric focusing

The procedure described by Vesterberg[9] was used with a LKB 8101 Ampholyne column and LKB carrier ampholytes. The time for an run was about 90 h and the temperature was maintained at 10 °C.

Gel filtration

This was carried out on a column (2.1 cm × 100 cm) of Sephadex G-75 (Pharmacia, Uppsala, Sweden) in 0.1 M NH_4HCO_3. The column was calibrated with yeast enolase (molecular weight, 80 000 (ref. 10); prepared according to Malmström[11]), human carbonic anhydrase B (mol. wt, 28 850 (ref. 12); prepared according to Henderson and Henriksson[6]), and horse heart cytochrome *c* (mol. wt, 12 400 (ref. 13); Sigma Type VI). The void volume, V_v, was determined with high molecular weight blue Dextran (Pharmacia) and was 99 ml. The proteins were eluted at the following volumes: enolase, 1.20 V_v; human carbonic anhydrase B, 1.72 V_v, and cytochrome *c*, 2.18 V_v.

Polyacrylamide gel electrophoresis

This was carried out as described by Smith[14] except that spacer and sample gels were omitted. The samples (about 75 μg) were applied in a 40% (w/v) sucrose solution (50 μl). The gels were prepared from 7.5% (w/v) acrylamide, and 0.095 M Tris–glycine buffer (pH 9.5), was used in both gel and electrode reservoirs. Gels were stained for protein overnight with amido black in 10% trichloroacetic acid, and destained in 10% acetic acid containing Amberlite resin MB 3.

Ultracentrifugation

Sedimentation velocity experiments were performed with a Beckman–Spinco Model E ultracentrifuge equipped with schlieren optics. The rotor speed was 59 780 rev./min. Exposures were taken every 16 min for 3 h. The sedimentation constants were calculated and corrected to give values in water at 20 °C ($s_{20,w}$) according to Svedberg and Pedersen[15].

Determination of amino acid composition

Amino acid analyses were carried out according to Moore and Stein[16] on a Beckman Model 120 B or a Beckman Unichrom automatic amino acid analyzer. The protein samples were hydrolyzed at 110 °C in 6 M HCl for 24, 48, and 72 h in tubes sealed *in vacuo* (50 μm). At least five samples were analyzed for each hydrolysis time. Half-cystine and methionine were estimated as cysteic acid and methionine sulfone, respectively, after performic acid oxidation according to Hirs[17]. Tryptophan was determined after hydrolysis in 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole according to the procedure of Liu and Chang[18], and the yields were corrected for destruction of tryptophan by extrapolation to zero hydrolysis time. Tryptophan was also estimated by the spectrophotometric method of Spies and Chambers[19].

The amino acid composition was calculated by a method similar to that used by Nyman and Lindskog[20]. This method is based on finding the molecular weight which gives a minimal deviation from integral values of the estimated numbers of amino acid residues per molecule. The results from each analysis were normalized by dividing by the alanine content. For the further calculations the average normalized values from all analyses were used, except for valine and isoleucine where only the 72 h data were used, and for serine and threonine, where the values were extrapolated to zero hydrolysis time assuming first-order kinetics for the destruction[16]. A Hewlett-Packard calculator 9100 B was used to obtain the sum, $s = \sum_i (|\, x_i f - n_i \,|/n_i)$, where x_i is the

normalized content of residue i, and n_i is the integer closest to $x_i f$. All amino acids except methionine were used in the summation. The factor f was varied in the range corresponding to molecular weights near 30 000 until a distinct minimum of s was found.

Determination of the molar absorbance coefficient

The absorbance coefficient of the protein was estimated after amino acid analysis of an enzyme sample with known absorbance.

Zinc analysis

Zinc analysis was performed with a Perkin–Elmer Model 403 atomic absorption spectrometer. Quartz-distilled water and acid-cleaned glassware were used. To remove metal ion impurities the enzyme samples were dialyzed overnight against 10^{-4} M EDTA (pH 7.1), followed by dialysis against quartz-distilled water. Zinc acetate solutions (5–40 μM) were used for calibration. Protein concentrations were estimated spectrophotometrically and by amino acid analysis and were approx. 25 μM in all cases.

Circular dichroism

CD spectra were measured on a Cary Model 60 spectropolarimeter equipped with a Cary 6002 CD accessory. From the recorded spectra the mean residue molecular ellipticities, $[\Theta]$, were calculated assuming a mean residue weight of 115.

Carboxyl terminal sequence

The protein was denatured by boiling for 15 min in 0.1 M $NaHCO_3$. Digestion was initiated at 37 °C by the addition of carboxypeptidases A and B (both DFP treated; Worthington, Freehold, N.J.), each in 1:100 weight ratio to carbonic anhydrase. Blank samples without carbonic anhydrase and carboxypeptidases, respectively, were run in parallel. Aliquots were withdrawn at intervals and incubated into sample dilution buffer for amino acid analysis and kept frozen until the analysis was carried out.

Isolation and partial sequence determination of tryptic peptide

A tryptic peptide from the major forms of moose carbonic anhydrase was isolated by the same procedure as that which has been used for the homologous peptide (residues number 90–113 (ref. 12)) from the human B enzyme[21]. After tryptic digestion of the whole protein the insoluble fraction was dissolved in 1 M acetic acid and subjected to gel filtration on Sephadex G-25 (fine) in 1 M acetic acid. The desired peptide was detected at a similar position in the elution pattern as the corresponding peptide from the human B enzyme. The peptide was further purified by gel filtration on Sephadex G-25 in 1 M acetic acid containing 2% dodecylamine. The amino terminal sequence was determined by sequential Edman degradation including dansylation according to Gray[22].

RESULTS

Enzyme purification

The yields of carbonic anhydrase activity during the purification procedure are

TABLE I

PURIFICATION OF CARBONIC ANHYDRASE FROM MOOSE ERYTHROCYTES

CO_2 hydration activity was measured by the colorimetric method described by Rickli *et al.*[7]. Concentrations of the purified enzymes were determined by absorbance measurements at 280 nm. The amount of starting material was 1.3 l of hemolysate. For details of the various purification steps see Materials and Methods.

Purification step	*Total activity (units × 10^{-7})*	*Spec. act. (units/mg × 10^{-4})*	*Yield (%)*
1. Hemolysate	4.16		100
2. Ethanol–chloroform extract	3.54		85
3. DEAE-cellulose chromatography:			
major form	2.23	10.6	54
minor form	0.34	6.3	8
4. Re-chromatography:			
minor form	0.30	6.8	7

shown in Table I. The crude extract obtained after treatment with a chloroform–ethanol–water mixture was further purified by chromatography on DEAE-cellulose. The results of a typical experiment are shown in Fig. 1. Two peaks containing CO_2 hydration activity were eluted, while other ultraviolet-absorbing material showed no detectable carbonic anhydrase activity. The main peak, which is eluted with 0.04 M Tris–HCl buffer (pH 8.7) and is centered around Fraction 80 (denoted major form), exhibits an approximately constant specific activity across the peak. In the second active peak, centered around Fraction 125 (denoted minor form), the specific activity

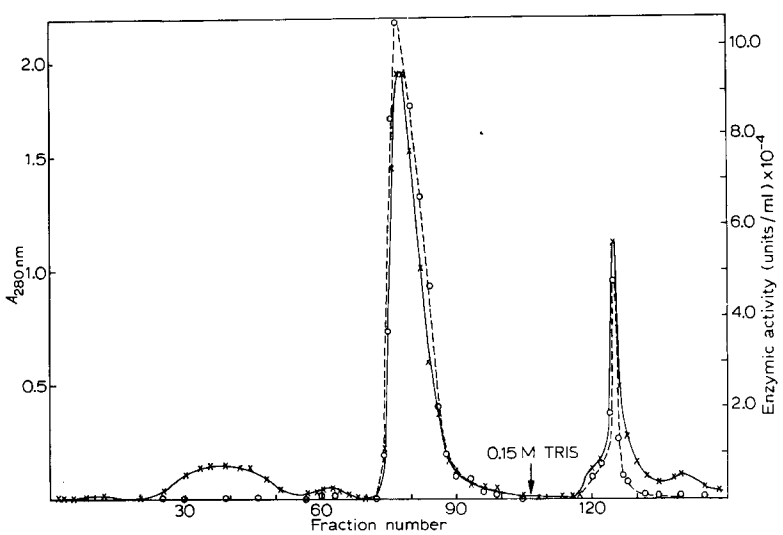

Fig. 1. Chromatography of crude moose carbonic anhydrase (ethanol–chloroform fraction) on a DEAE-cellulose column (2.8 cm × 45 cm) equilibrated with 0.01 M Tris–HCl buffer (pH 8.7). Elution was performed with 0.04 M Tris–HCl buffer (pH 8.7), and from Fraction 107 with 0.15 M Tris–HCl buffer (pH 8.7). After Fraction 150 the column was washed with 0.5 M Tris–HCl buffer (pH 8.7). Fraction volumes were 26 ml and the flow rate was held constant by a peristaltic pump at 0.65 ml/min. Temperature was 4 °C. ×—×, $A_{280\,nm}$; ○– –○, CO_2 hydration activity in units/ml. Fractions 73–90 were pooled and represent the major form, while Fractions 117–133, containing the minor form, were re-chromatographed as described in the text.

is not uniform through the peak and some impurities are evidently still present. The inhomogeneity of this fraction was also revealed by polyacrylamide gel electrophoresis. Two contaminant bands, constituting approx. 10% of the total material, were observed. A total of 73% of the carbonic anhydrase activity loaded onto the column was eluted in the fractions containing the major and minor forms. Washing the column with 0.5 M buffer did not result in any further elution of material having CO_2 hydration activity.

The fractions from the DEAE-cellulose chromatography containing the minor carbonic anhydrase form were pooled and rechromatographed on the same DEAE-cellulose column. The enzyme was eluted with 0.06 M Tris–HCl buffer (pH 8.7) and appeared as a symmetrical peak with approximately constant specific activity. No other active material was eluted. The minor form appears to have a somewhat lower specific activity than the major form even after rechromatography.

The total yield of moose carbonic anhydrase from 1.3 l of hemolysate was 210 mg of the major form and 50 mg of the minor form.

Criteria of purity and some physical properties

The major and minor forms appeared homogeneous on polyacrylamide gel electrophoresis as illustrated in Fig. 3.

The two forms were examined separately by isoelectric focusing in the pH region

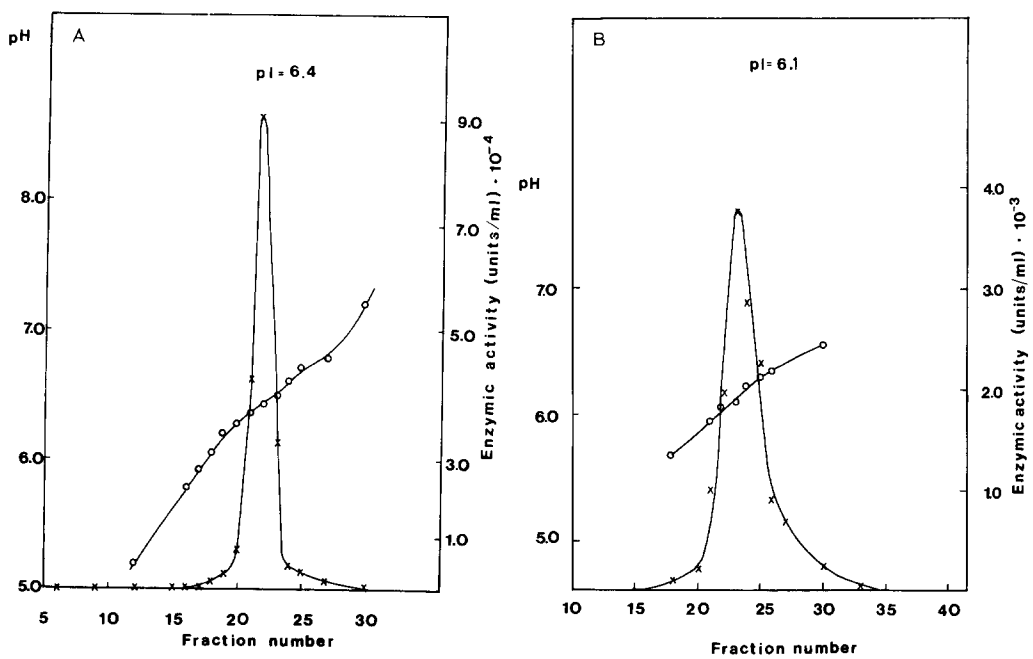

Fig. 2. Isoelectric focusing of moose carbonic anhydrase: (A) the major form from the DEAE-cellulose chromatography, (B) the minor form from the DEAE-cellulose re-chromatography. The pH gradient was 5–8 with the anode at the acidic region. The focusing was carried out for 90 h at 10 °C. Fraction volumes of 2.8 ml were collected by means of a peristaltic pump working at a constant rate of 0.7 ml/min. The circles (○) show the pH of the fractions and the crosses (×) the CO_2 hydration activity in units/ml.

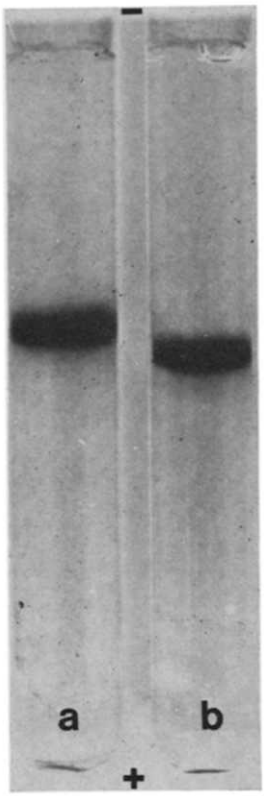

Fig. 3. Acrylamide gel electrophoresis of moose carbonic anhydrase: (A) the major form from the DEAE-cellulose chromatography, (B) the minor form from the DEAE-cellulose re-chromatography. The electrophoresis was carried out as described in Materials and Methods.

5–8. The results given in Fig. 2 indicate that the minor form may be slightly contaminated with the other form, while the major form appears in a narrow, symmetrical band of activity. The isoelectric points are given in Table II. To investigate if any form of moose carbonic anhydrase has been missed during the purification,

TABLE II

PHYSICAL PROPERTIES OF CARBONIC ANHYDRASE FROM MOOSE AND OX

	Moose		*Ox, Major form (B)*
	Minor form	*Major form*	
Sedimentation constant $s_{20,w}$ (Svedberg units)*		2.78	2.85 (ref. 2)
Molecular weight** $\times 10^{-3}$	29.8	29.2	29.5 (ref. 20)
Isoelectric pH	6.1	6.4	5.9 (ref. 23)
$\varepsilon_{280\ nm} \times 10^{-3}$ ($M^{-1} \cdot cm^{-1}$)***	61.5	56.7	57.0 (ref. 20)

* Enzyme concentration 7.0 mg/ml.
** Calculated from amino acid composition.
*** Calculated using a mol. wt of 30 000.

isoelectric focusing was also performed on the crude chloroform–ethanol extract in a pH gradient between pH 3 and 10. Only two peaks of CO_2 hydration activity were detected having isoelectric points corresponding to those of the purified forms.

Each form was eluted as a single, symmetrical peak after gel filtration on Sephadex G-75. The major form appeared after 1.75 void volumes and the minor form after 1.76 void volumes. Using enolase, human carbonic anhydrase B, and cytochrome *c* to construct a calibration curve, log (mol. wt) = *f* (elution volume), these elution volumes would correspond to molecular weights of about 27 500 for the moose isoenzymes.

The major moose form (7.0 and 11.5 mg/ml) sedimented as a single, symmetrical boundary throughout the run (3 h) in the analytical ultracentrifuge. The sedimentation coefficient is given in Table II together with some other properties of moose carbonic anhydrase.

The CD spectrum of the major moose form is shown in Fig. 4 together with that of the major form of bovine carbonic anhydrase.

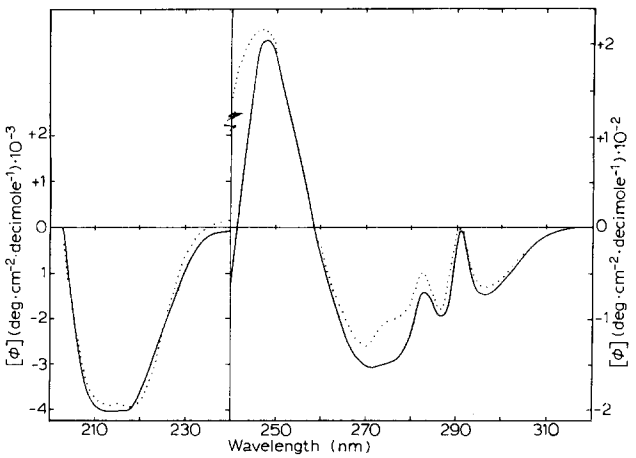

Fig. 4. CD spectra of the major forms of moose carbonic anhydrase (solid line) and bovine carbonic anhydrase (B) (dotted line). CD measurements from 320 to 240 nm were made in 1-cm quartz cells at an enzyme concentration of about 1 mg/ml. Below 240 nm 1-mm quartz cells were used in an unbuffered solution, pH 7.9. Temperature was 25 °C.

Composition

In Table III the amino acid compositions of the two forms of moose carbonic anhydrase are given together with the composition of the major form of the bovine enzyme. Zinc analysis was performed by atomic absorption spectrometry on duplicate samples. The major and minor forms contained 0.217 and 0.237% zinc, respectively, corresponding to 1.00 and 1.09 atoms of zinc per 30 000 g of enzyme.

Esterase activity

The pH rate profile of the esterase activity of the major form with *p*-nitrophenyl acetate as substrate is shown in Fig. 5.

TABLE III

AMINO ACID COMPOSITIONS OF CARBONIC ANHYDRASES FROM MOOSE AND OX

The amino acid composition of moose carbonic anhydrase was estimated as described in Materials and Methods.

Residue	*Moose*				*Ox, Major form**
	Minor form		*Major form*		
	Observed	*Integer*	*Observed*	*Integer*	
Trp**	7.9	8	7.0	7	7
Lys	20.4	20	20.4	20	19
His	10.9	11	11.0	11	11
Arg	9.1	9	9.2	9	9
Asp	34.1	34	33.4	33	32
Thr	12.9	13	12.9	13	15
Ser	19.0	19	19.2	19	16
Glu	21.7	22	21.5	22	24
Pro	20.7	21	20.0	20	20
Gly	19.6	20	19.4	19	20
Ala	19.4	19	19.2	19	17
Val	20.0	20	20.3	20	20
Met***	2.6	3	2.9	3	3
Ile†	1.1	1	1.0	1	5
Leu	27.9	28	26.9	27	26
Tyr	6.2	6	6.0	6	8
Phe	12.0	12	11.7	12	11
Cys†,††	<0.1	0	<0.1	0	0
		266		261	263

* Values taken from Nyman and Lindskog[20].

** The spectrophotometric method of Spies and Chambers[19] gave 6.9 residues for the major form.

*** Unoxidized samples gave the same result as treatment with performic acid according to Hirs[17].

† Samples of high protein concentration were analyzed in order to obtain better accuracy.

†† Determined as cysteic acid after performic oxidation according to Hirs[17].

Carboxyl terminal sequence

During digestion of the major form of the moose enzyme with a mixture of carboxypeptidases A and B arginine was released most rapidly followed by leucine. After a 4-h digestion the yields of arginine and leucine were 0.99 and 0.48 mole per mole of enzyme, respectively, while the yields of all other amino acids were less than 0.10 mole per mole of enzyme. Proline is the penultimate residue at the carboxy-terminals of bovine[24] and sheep[3] carbonic anhydrases, but no proline was detected even after a 13-h digestion of the moose enzyme. Thus, the proposed carboxyl terminal sequence of the major form of moose carbonic anhydrase is –Leu–Arg.

Partial sequence of tryptic peptide

The prepared tryptic peptide had the following amino acid composition: Lys, 2.0 (2); His, 3.3 (3); Asp, 2.9 (3); Thr, 0.95 (1); Ser, 2.9 (3); Glu, 3.0 (3); Gly, 2.3 (2); Val, 2.1 (2); Leu, 1.2 (1); Phe, 1.8 (2); Trp (1). The numbers within paranthesis are the nearest integral values. The tryptophan content was estimated from the ultraviolet absorption of the peptide. The tryptic peptide from the moose enzyme consists of 23 residues, while the homologous peptides from the human B and C

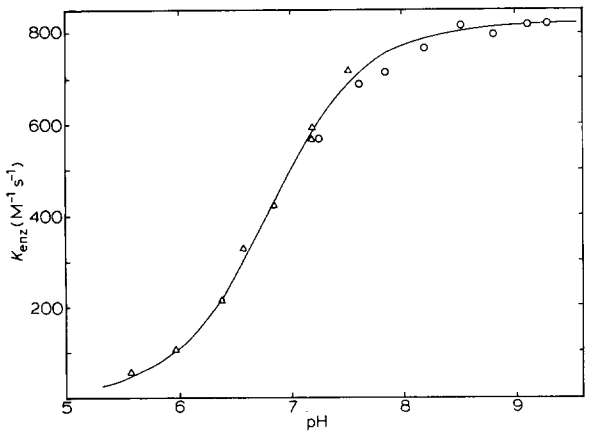

Fig. 5. pH–rate profile for the hydrolysis of *p*-nitrophenyl acetate catalyzed by moose carbonic anhydrase (major form). The assay mixture contained 4% (v/v) acetone, 0.4 mM *p*-nitrophenyl acetate, 0.78 or 1.56 μM enzyme. Temperature, 25 °C. Triangles (△) denote *N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane–H_2SO_4 buffers and circles (○) Tris–H_2SO_4 buffers, all of ionic strength 0.1. The substrate concentration is much smaller than K_m. The apparent second-order rate constant, k_{enz}, is defined by the relation $v_0 = k_{enz} \cdot [E] \cdot [S]$, where v_0 is the inital rate (M/s) of the catalyzed reaction. The curve has been calculated assuming that the basic forms of a group with a pK value of 6.8 represents the active enzyme, and that k_{enz} approaches 820 $M^{-1} \cdot s^{-1}$ at alkaline pH.

enzymes contain 24 and 23 residues, respectively[12,21,25]. Edman degradation by the dansyl method gave the following amino terminal sequence: Leu–Val–Glx–Phe–His–Phe–His–(Trp)–Gly–. Dansyl tryptophan is decomposed during acid hydrolysis, and the paranthesis indicates that no dansyl amino acid was detected from the corresponding Edman step. From this result and homology considerations[12,25] we infer that tryptophan occupies this position.

DISCUSSION

The two isolated forms of moose erythrocyte carbonic anhydrase both have specific CO_2 hydration activities of a magnitude that is characteristic for the high activity (C-type) mammalian carbonic anhydrases. The serine content (19 residues per molecule) is within the range observed for C-type enzymes (16–22 residues per molecule[1]), whereas the B-type carbonic anhydrases from a variety of species show a much higher serine content (28–33 residues per molecule[1]). Thus, both forms of the moose enzyme are C-type carbonic anhydrases also by this criterion.

The minor form may either represent a genetic variant or a secondary modification of the major form. The present data do not allow a definite distinction between these possibilities. The amino acid compositions of the two forms are almost identical. The difference in tryptophan content (and absorbance coefficient) appears to be significant, but the presence of small amounts of a tryptophan-rich impurity in the preparations of the minor form cannot be excluded. The isoenzyme composition of moose carbonic anhydrase is very similar to that of the bovine erythrocyte enzyme. In this case evidence was recently presented that two forms, originally designated B and A (ref. 2), differ by an Arg→Glu interchange[26]. A third bovine form, present in

very small amounts, appears to be a secondary modification of the major form[26].

We did not detect any B-type moose carbonic anhydrase. Thus, it appears that the red cells of the moose, like the red cells of all other ruminants studied so far, lack a low-activity isoenzyme. The presence in moose blood of a B form with an unusually high isoelectric point (*cf.* the horse C enzyme[27]) seems unlikely in view of the results of the isoelectric focusing experiment in which a pH gradient between 3 and 10 was employed. In the case of the bovine enzyme we have investigated the possibility that a low-activity form has been missed during the preparation, for example through selective denaturation during the ethanol–chloroform treatment. However, we could not detect any low-activity isoenzyme despite extensive fractionation of bovine hemolysate on a variety of ion-exchange resins.

The sequence homologies shown by the human B and C enzymes[1,12,25] suggest that the differentiation between the isoenzyme types originates in the duplication of an ancestral carbonic anhydrase gene and a subsequent independent evolution. Since both enzyme types have been found in species (man[28], pig[29], horse[27], guinea-pig[30]) representing several mammalian orders, the gene duplication apparently occurred before these orders diverged. The demonstration by Carter[31] of a B-type carbonic anhydrase in the rumen epithelial tissue of the ox suggests that, at least in this ruminant, the absence of an erythrocyte B enzyme is due to gene repression rather than to a lack of the corresponding gene (*cf.* Ashworth *et al.*[4]).

As might be expected from the evolutionary relationships, the physical and chemical properties of the moose enzyme are very similar to those of other mammalian carbonic anhydrases, particularly the bovine and sheep enzymes (Table II). The CD spectra of the moose and ox enzymes show only small differences in intensities of some of the bands (Fig. 4). Presumably the tertiary structures of these enzymes are closely similar. With *p*-nitrophenyl acetate as substrate the moose enzyme has a slightly lower specific activity than the bovine enzyme[8], while the human C enzyme is considerably more active with this substrate[32]. The pH–rate profile is sigmoidal and characterized by a pK value of 6.8. The corresponding pK value for the bovine enzyme is 6.9 (ref. 8).

As noted by Ashworth *et al.*[4] the carbonic anhydrases from erythrocytes of ruminants have a distinctly lower isoleucine content than the enzymes from other investigated species. The moose enzyme, having a single isoleucine residue, conforms to this pattern. In addition, the moose enzyme, like the enzymes from other ruminants and pig (order *Artiodactyla)*, is devoid of cysteine.

Although the amino acid compositions of moose and ox carbonic anhydrases (Table III) are quite similar, the data indicate that the sequences might differ in at least 20 positions. The limited sequence information obtained for the moose enzyme illustrates such differences as well as some similarities. The amino terminal part of the isolated tryptic peptide represents one of the most constant regions in the carbonic anhydrases. In the human C enzyme histidine residues 93 and 95 of the corresponding peptide are involved as ligands to the metal ion in the active site[1]. The carbonic anhydrases from ox[33], sheep (Tashian, R. E., personal communication), and moose appear to display the identical sequences –Leu–Val–Gln–Phe–His–Phe–His–Trp–Gly–, while the corresponding sequences of the human B and C enzymes differ only by having phenylalanine[12] or isoleucine[25], respectively, at the position of valine in the ruminant enzymes.

The carboxyl terminal 6 or 7 residues of the peptide chain constitute one of the most variable regions in the carbonic anhydrases. The C-terminals of previously studied ruminant enzymes are either –Pro–Lys (ox[24], sheep[3]) or –Pro–Arg (deer[4]), while the human and horse C enzymes terminate with the sequence –Phe–Lys[24,4]. Thus, the C-terminal of the moose enzyme, –Leu–Arg, differs in both positions from the terminals of the bovine and sheep enzymes. It was pointed out by Nyman *et al.*[24] that a Pro↔Phe interchange at the penultimate position would require two base changes in the corresponding codon. It is interesting to note that both a Leu↔Pro interchange and a Leu↔Phe interchange only require single-base changes.

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REFERENCES

1 Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O. and Strandberg, B. (1971) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn, Vol. 5, pp. 587–665, Academic Press, New York
2 Lindskog, S. (1960) *Biochim. Biophys. Acta* 39, 218–226
3 Tanis, R. J. and Tashian, R. E. (1971) *Biochemistry* 10, 4852–4858
4 Ashworth, R. B., Brewer, J. M. and Stanford, Jr, R. L. (1971) *Biochem. Biophys. Res. Commun.* 44, 667–674
5 Keilin, D. and Mann, T. (1940) *Biochem. J.* 34, 1163–1176
6 Henderson, L. E. and Henriksson, D. (1973) *Anal. Biochem.* 51, 288–296
7 Rickli, E. E., Ghazanfar, S. A. S., Gibbons, B. H. and Edsall, J. T. (1964) *J. Biol. Chem.* 239, 1065–1078
8 Thorslund, A. and Lindskog, S. (1967) *Eur. J. Biochem.* 3, 117–123
9 Vesterberg, O. (1971) in *Methods in Enzymology* (Jakoby, W. B., ed.), Vol. 22, pp. 389–412, Academic Press, New York
10 Harris, C. E., Kobes, R. D., Teller, D. C. and Rutter, W. J. (1969) *Biochemistry* 8, 2442–2454
11 Malmström, B. G. (1957) *Arch. Biochem. Biophys.* 70, 58–69
12 Andersson, B., Nyman, P. O. and Strid, L. (1972) *Biochem. Biophys. Res. Commun.* 48, 670–677
13 Margoliash, E. (1962) *J. Biol. Chem.* 237, 2161–2174
14 Smith, J. (1968) *Chromatographic and Electrophoretic Techniques*, Vol. 2, 2nd edn, pp. 365–418, Heinemann, London
15 Svedberg, T. and Pedersen, K. O. (1940) *The Ultracentrifuge*, Oxford University Press, New York
16 Moore, S. and Stein, W. (1963) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 6, pp. 819–831, Academic Press, New York
17 Hirs, C. H. W. (1967) in *Methods in Enzymology* (Hirs, C. H. W., ed.), Vol. 11, pp. 59–62, Academic Press, New York
18 Liu, T.-Y. and Chang, Y. H. (1971) *J. Biol. Chem.* 246, 2842–2848
19 Spies, J. R. and Chambers, D. C. (1949) *Anal. Chem.* 21, 1249–1266
20 Nyman, P. O. and Lindskog, S. (1964) *Biochim. Biophys. Acta* 85, 141–151
21 Strid, L. (1973) *FEBS Lett.* 33, 192–196
22 Gray, W. R. (1967) in *Methods in Enzymology* (Hirs, C. H. W., ed.), Vol. 11, pp. 469–475, Academic Press, New York
23 Jonsson, M. and Pettersson, E. (1968) *Acta Chem. Scand.* 22, 712–713
24 Nyman, P. O., Strid, L. and Westermark, G. (1968) *Eur. J. Biochem.* 6, 172–189
25 Henderson, L. E., Henriksson, D. and Nyman, P. O. (1973) *Biochem. Biophys. Res. Commun.* 52, 1388–1394
26 Charrel, M., Gulian, J., Laurent, G. and Derrien, Y. (1973) *C. R. Sér. D* 276, 1061–1063

27 Furth, A. J. (1968) *J. Biol. Chem.* 243, 4832–4841
28 Nyman, P. O. (1961) *Biochim. Biophys. Acta* 52, 1–12
29 Tanis, R. J., Tashian, R. E. and Yu, Y. S. L. (1970) *J. Biol. Chem.* 245, 6003–6009
30 Carter, M. J. and Parsons, D. S. (1970) *Biochem. J.* 120, 797–808
31 Carter, M. J. (1971) *Biochim. Biophys. Acta* 235, 222–236
32 Verpoorte, J. A., Mehta, S. and Edsall, J. T. (1967) *J. Biol. Chem.* 242, 4221–4229
33 Laurent, G., Foveau, D., Limozin, N. and Sciaky, M. (1973) *C. R. Sér. D* 276, 827–830