Composition and Carboxyl-Terminal Amino Acid Sequences of Some Mammalian Erythrocyte Carbonic Anhydrases

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Received June 14, 1971

Summary: Carbonic anhydrases (carbonate hydrolyase, E. C. 4.2.1.1) were isolated from goat, sheep and deer erythrocytes and their amino acid compositions determined. Carboxyl-terminal sequences of these and other mammalian erythrocyte carbonic anhydrases were determined by digestion with carboxypeptidases A and B and hydrazinolysis. The data suggest that gene duplication produced the lower specific activity ("B form") carbonic anhydrase from the higher specific activity ("C type") enzyme after the ancestor of the goat, sheep, deer and cow diverged from the ancestor of the pig, horse, monkey and human. In addition, it is suggested that the B-type carbonic anhydrase is simply a partial replacement to the C-type enzyme and has no special physiological significance.

Materials and Methods

Pig and cow blood were obtained at a local slaughterhouse. Horse, goat and sheep blood were donated by Dr. C. W. Foley of the University of Georgia School of Veterinary Medicine. Deer blood samples were donated by Dr. T. P. Kistner of the Southeastern Cooperative Wildlife Disease Survey. DEAE-cellulose was purchased from the Reeve Angel Co. and carboxypeptidases A and B came from the Worthington Chemical Co.

Chromatography and isoelectric focusing were done as described in reference 1. Analytical and preparative disc electrophoresis was done as described in reference 2. The enzyme was assayed by the method of Wilbur and Anderson (3) as modified by Rickli *et al.* (4). Determination of amino acids was made with a Beckman Model 120C Amino Acid Analyzer. Measurements of protein were made by dry weight of lyophilized material, assuming a moisture content of 15%, or from the absorbance at 280 nm of solutions of carbonic anhydrases, assuming an extinction coefficient of 1.74 (1). Analyses were made of protein samples which had been hydrolyzed in 6N HCl as described by Spackman *et al.* (5). Tryptophan,

half-cystine and methionine were determined by the methods of Matsubara and Sasaki (6) and of Hirs (7).

Carboxyl-terminal sequences were determined as described in reference 1, except that trichloroacetic acid was used to denature the carbonic anhydrases before digestion. Hydrazinolysis was done according to Braun and Schroeder (8).

The carbonic anhydrases were isolated from erythrocytes of goat, deer, cow, sheep, horse and pig essentially by the method of Ashworth *et al.* (1), which employs elution from a DEAE-cellulose column with a very shallow ionic strength gradient. Further purification of the goat, sheep and deer enzymes was done by isoelectric focusing (9) or preparative disc electrophoresis at pH 9.0 (2). The isolated enzymes were analyzed for purity by analytical disc electrophoresis at several pH's. They were estimated from the disc electrophoresis patterns to be 90-100% homogeneous in all cases (1).

Results and Discussion

Carbonic anhydrase occurs in two major forms in the erythrocytes of several mammals. These forms differ in specific activity and amino acid composition (10). The relative amounts of the two major forms found in each of the mammalian sources investigated is given in Table I. These percentages are listed along with the specific activities of each form and the total units of carbonic anhydrase per volume of red cells from several species. The carbonic anhydrases are classified as B or C types on the basis of their specific activities, as suggested by Edsall (15).

While the specific activities in the case of horse, pig and monkey carbonic anhydrases are lower than those of the others, this may be due to interfacial adsorption of the protein during the assay. This is certainly the case with the porcine B enzyme (1), and could be the case with horse and monkey carbonic anhydrases, since those who isolated these enzymes apparently did not check the possibility that such adsorption might be occurring (10,12). It is therefore possible that the carbonic anhydrases described here all have specific activi-

TABLE I. RELATIVE AMOUNTS AND SPECIFIC ACTIVITIES OF B AND C-TYPE CARBONIC ANHYDRASES FROM ERYTHROCYTES OF SEVERAL MAMMALS

Source	Reference	Carbonic Anhydrase Form, percent of total weight in erythrocytes		Ratio, B/C	Specific Activity, units/mg.		Total Carbonic Anhydrase Activity, units/liter of packed erythrocytes
		B-type	C-type		B-type	C-type	
human	11	86*	12	7.2	16,000	60,000	$3 \cdot 10^7$ **
monkey	12	87	13	6.7	10,500	32,000	-
pig	1,***	60	23	2.6	13,700	55,000	$5 \cdot 10^7$ ***
horse	10,***	64	36	1.8	3,600	34,800	-
cow	13,***	0	100*	0.0	-	60,000	$2 \cdot 10^7$
sheep	***	0	100****	0.0	-	60,000	-
deer	***	0	100	0.0	-	62,000	$6 \cdot 10^7$ ***
goat	***	0	100	0.0	-	60,000	-

* Including the "A" enzyme, which has the same specific activity as the "B" form. The bovine "A" and "B" enzymes are high specific activity forms, so are relabeled. The bovine "A" form is believed to be a deamidated form of the so-called "B" enzyme.

** Calculated from data in reference 11.

*** This work.

**** A small quantity of a second form with an apparently lower specific activity was isolated from DEAE columns, but on purification by isoelectric focusing was found to consist of at least two components with lower isoelectric points than the C enzyme and specific activities approaching that of the C enzyme. In addition, their amino acid compositions were similar to that of the C enzyme. We conclude that these minor active proteins are not of the B type but are probably deamidated forms of sheep carbonic anhydrase C. A similar conclusion was reached by Tanis and Tashian (14).

TABLE II. AMINO ACID COMPOSITION OF MAMMALIAN ERYTHROCYTE CARBONIC ANHYDRASES*

Amino Acid	Deer C	Goat C**	Sheep C	Cow B(C)	Horse B	Pig B	Monkey B	Human B	Horse C	Pig C	Monkey C	Human C
Tryptophan	5	5	6	7	5	6	7	6	7	6	7	7
Lysine	20	20	19	19	19	23	18	18	19	20	24	25
Histidine	13	12	12	11	10	14	9	11	12	13	12	12
Arginine	11	10	10	9	5	6	7	7	9	9	8	7
Aspartate	32	32	32	32	32	34	36	31	27	27	30	29
Threonine	13	12	13	15	12	11	13	14	12	13	11	13
Serine	17	18	18	16	28	29	30	30	18	17	18	19
Glutamate	23	26	27	24	25	21	22	22	26	28	26	24
Proline	21	21	21	20	18	17	17	17	16	17	18	18
Glycine	21	21	18	20	23	18	15	16	23	22	22	22
Alanine	20	24	19	17	15	20	16	19	17	13	12	13
Half-Cystine	0	0	0	0	2	0	1	1	1	0	1	1
Valine	22	23	23	20	20	16	16	17	19	13	17	17
Methionine	2	3	3	3	2	0	1	2	1	3	2	1
Isoleucine	2	5	3	5	9	15	10	9	7	10	9	9
Leucine	26	28	28	26	21	23	19	20	22	23	27	27
Tyrosine	7	7	7	8	8	11	9	8	7	7	7	9
Phenylalanine	13	13	12	11	11	9	10	11	11	12	13	13

*These data are taken from: bovine B(C), reference 16; horse B and C, reference 10; porcine B, reference 1; monkey B and C, reference 12; human B and C, reference 17; porcine C, reference 18. The number of residues in the deer, sheep and goat enzymes are calculated from the averages of 24, 48 and 72 hour acid hydrolyzates of the native proteins, except that the values for serine and threonine were obtained by extrapolation to zero time of hydrolysis, the values for valine and isoleucine by extrapolation to infinite time of hydrolysis, methionine and half-cystine from 24 hour acid hydrolyzates of performic acid oxidized protein (7) and tryptophan was found after 24 hours of hydrolysis in the presence of 6 N HCl containing 4% thioglycolic acid (6). 0.3-1.0 mg of protein was hydrolyzed for each time point and 1.5 mg for the performic acid samples. The tryptophan value is corrected, assuming 85-90% recovery (6). Half-cystine contents (as cysteic acid) were less than 0.1 moles/mole of protein. Analyses of the sheep enzyme were run in triplicate; for the sheep, deer and goat enzymes the average variation between analyses was less than 5%.

**The amino acid composition of the goat enzyme was calculated assuming three methionines in the enzyme.

ties of 55,000-62,000 for the C form and 13,700-18,000 for the B form.

The relative percentages of B and C forms vary widely. Indeed, the animals appear to fall into two distinct groups based on whether their erythrocytes contain predominantly the B form (human, monkey, horse and pig) or wholly the C form (cow, sheep, deer and goat).

The amino acid compositions of the goat, deer and sheep carbonic anhydrases are shown in Table II, together with previously published compositions of other enzymes (1,10,12,16,17,18). While there is considerable variation between species, two correlations can be seen. The C-type enzymes from the deer, sheep, goat and cow have lower isoleucine contents than the B and C-type enzymes from the horse, pig, monkey and human. In addition, the B-type enzymes from the latter group are characterized by a considerably higher serine content.

The carboxyl-terminal sequences of several carbonic anhydrases were determined by digestion with carboxypeptidases A and B and by hydrazinolysis of proteins which were resistant to further hydrolysis by carboxypeptidases. Table III gives the results of such digestions on the carbonic anhydrases from the sheep, horse, pig and goat enzymes, together with the corresponding sequences already known from previous work (1,14,19).

With the deer, sheep and bovine carbonic anhydrases, only one mole of lysine or arginine is released by carboxypeptidase B. This suggested that the next residue was aspartic or glutamic acid or proline. In order to demonstrate the presence of proline in the penultimate position in the deer and sheep enzymes (where it occurs in the bovine), these proteins were digested with carboxypeptidase B and then subjected to hydrazinolysis.

Proline was released in all cases with yields of 24-34%, based on the amount of carboxyl-terminal lysine or arginine released. Little or no aspartic or glutamic acid was released, suggesting that the penultimate residue in the deer and sheep carbonic anhydrases is proline.

The carboxyl-terminal sequences consequently fall into two groups: those with carboxyl-terminal prolyl-lysine or -arginine and those with phenylalanine as the carboxyl-terminal or penultimate residue.

TABLE III. CARBOXYL-TERMINAL SEQUENCES OF SOME MAMMALIAN CARBONIC ANHYDRASES*

Source	References	Carboxyl-terminal sequence
human B	19	-val-arg-ala-ser-phe
human C	19	-ileu-lys-ala-ser-phe-lys
porcine B	1	-lys-ala-ser-phe
porcine C	**	$\begin{matrix}\text{-arg}\\\text{-lys}\end{matrix}$-ser-phe
horse B	**	-val-arg-ala-phe-phe
horse C	**	-ileu-arg-ala-ser-phe-lys
bovine B(C)	19	-val-arg-gly-phe-pro-lys
sheep C	**,14	-val-arg-val-phe-pro-lys
deer C	**	-pro-arg

*The sequences were obtained in this work by analysis of the quantities of amino acids released with an amino acid analyser as a function of time. Denatured carbonic anhydrases were digested at 37° by diisopropylfluorophosphate (DFP)-treated carboxypeptidase A and in separate experiments by DFP-treated carboxypeptidase B. A zero time and 4-6 additional time points were taken in each experiment, using 0.05 μmoles of protein substrate for each time point. A weight ratio of 1/200 carboxypeptidase to denatured protein substrate was used. No amino acids were detected in the zero time or enzyme blank samples. Some typical experimental data are given below:

Substrate: horse carbonic anhydrase B.

Moles amino acid released per mole protein, using:

A. Time	carboxypeptidase A	B. Time	carboxypeptidase B (average of duplicate samples)
20 min	0.41 phe	20 min	0.59 phe
1 hrs	0.77 phe; 0.11 ala	1.5 hrs	0.76 phe; 0.23 arg; 0.23 ala
3 hrs	1.09 phe; 0.32 ala	6 hrs	1.01 phe; 0.44 arg; 0.42 ala; 0.22 val
9 hrs	1.53 phe; 0.59 ala	20 hrs	1.22 phe; 0.56 arg; 0.52 ala; 0.34 val

The hydrazinolyses were carried out on 0.2-0.6 μmoles of carboxypeptidase B digested protein at 80° for 20 hours.

**This work.

Our data are of course quite limited, although more extensive than any compiled so far. Consequently, our conclusions must be tentative.

A great deal of research and speculation has evolved from the observation that some species have two major forms of carbonic anhydrase with different specific activities. The feeling has been that the two forms must have different physiological functions. Our data, however, indicate that the two forms have the same function, since the total activity per volume of red cells is roughly constant from species to species despite widely varying ratios of B-type to C-type carbonic anhydrase. Furthermore, there is no correlation between the B/C ratio and such physiological parameters as basal metabolic rate (which is essentially a function of body size) (20), diet and overall activity. The horse and cow, for example, are grazing herbivores, but the carbonic anhydrase contents of their blood are quite different.

On the other hand, there is a striking correlation between the amino acid composition, carboxyl-terminal amino acid sequence and the B/C ratio, that is,

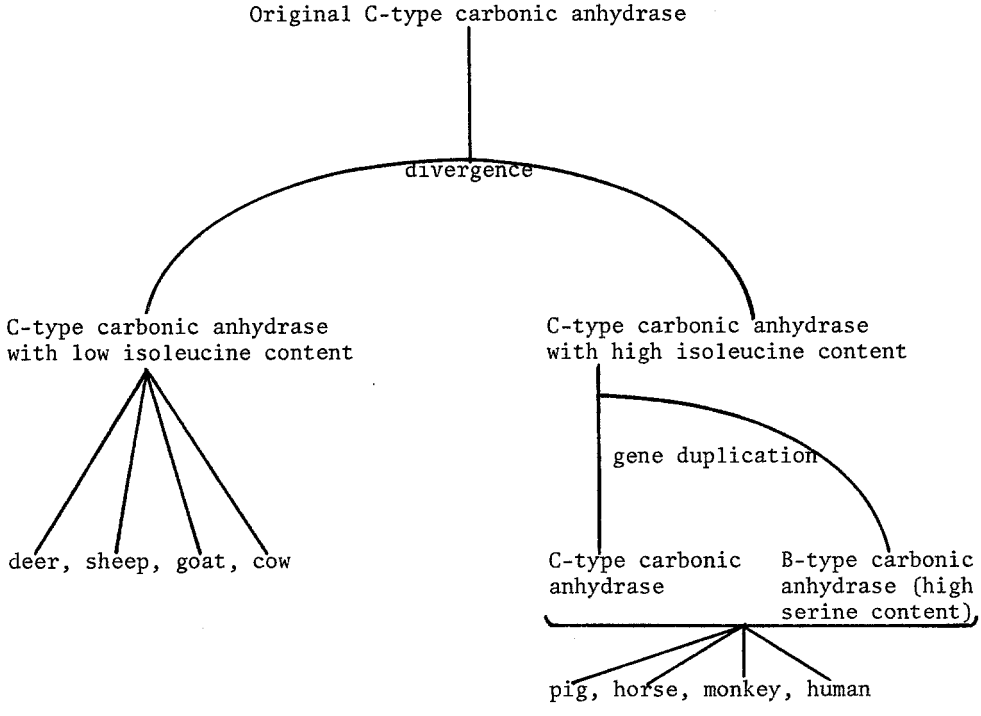

Figure 1. Hypothetical scheme of evolution of some mammalian species, based on amino acid compositions and carboxyl-terminal sequences of erythrocyte carbonic anhydrases.

between the evolutionary grouping and the predominant carbonic anhydrase type in the erythrocytes. Our interpretation of the above data is described in Figure 1. We speculate that the gene duplication producing the second (probably the B) form occurred before the ancestor of the goat, sheep, deer and cow (Bovidae and Cervidae families) diverged from the ancestor of the pig, horse, monkey and human.

Acknowledgements

The authors are obliged to the MacEvers Packing Co. of Talmo, Georgia and to Dr. C. W. Foley of the University of Georgia School of Veterinary Medicine for generous gifts of pork, cow, horse, sheep and goat blood and to Dr. T. P. Kistner of the Southeastern Cooperative Wildlife Survey for gifts of deer blood. This research was supported by grants from the National Science Foundation to R. B. Ashworth (GB 7771) and J. M. Brewer (GB 13031).

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