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CHARACTERISATION OF CARBONIC ANHYDRASES FROM TISSUES OF THE CAT

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Summary

1. Red blood cells from several members of the cat family have been found to contain only carbonic anhydrase (EC 4.2.1.1) isozymes of the "high activity" (carbonic anhydrase II class) in red blood cells.

2. Two carbonic anhydrase II type isozymes have been isolated from red cells of the domestic cat. Kinetic and structural characterisation shows that the two isozymes have identical composition except for a probable difference of one amide group. The general characteristics of cat red cell carbonic anhydrase are similar to other mammalian carbonic anhydrase II isozymes, in particular the dog carbonic anhydrase II.

3. A carbonic anhydrase I (low activity) isozyme has been isolated and characterised from cat caecal mucosa. This carbonic anhydrase I is similar to the carbonic anhydrase I extracted from canine red cells. Comparative studies showed that whereas members of the dog family have carbonic anhydrase I and carbonic anhydrase II in red cells the cat family has only a carbonic anhydrase II type isozyme, however, cat liver and spleen homogenates were also found to contain carbonic anhydrase I identical to that found in caecum.

Introduction

Carbonic anhydrase (EC 4.2.1.1) has been found to exhibit two genetically distinct isozymes in most mammalian erythrocytes [1].

Two primary carbonic anhydrase isozymes exhibiting high (carbonic anhydrase II) and low activity (carbonic anhydrase I) have now been demonstrated in dog erythrocytes [2]. This paper describes a comparative study of cat carbonic anhydrases, in particular the domestic cat, *Felis catus*.

Materials and Methods

Electrophoretic techniques. Electrophoresis was carried out on cellogel strips in a Tris/borate/EDTA buffer at pH 9.1 as described previously [3]. Gels were stained with nigrosin to locate protein zones. Specific activity stains for carbonic anhydrase using bromothymol blue were carried out as described previously [2,3].

Assays. CO_2 hydrase activity was measured using the bromothymol blue assay [4]. The carboxylic esterase was determined using *p*-nitrophenol acetate as substrate [5]. Fluorescein diacetate and umbelliferone acetate were used to assay specific esterase activity of the carbonic anhydrase I and carbonic anhydrase II isozymes [2].

Amino acid analysis. 0.1—1-mg samples of the salt-free enzyme were submitted to graded hydrolysis, at 110°C, in 6 M HCl. Amino acid analysis was carried out by using a Locarte analyser. Tryptophan was determined by oxidation with *N*-bromosuccinimide [6], and cysteine was measured as cysteic acid.

Reaction of isozymes with antiserum. The antiserum used was rabbit anti-human carbonic anhydrase I, prepared as previously described [7]. Reaction with antiserum was determined by diffusion in agar gels, followed by staining of precipitin bands with nigrosin.

Purification of carbonic anhydrase isozymes. (a) Blood: 100-ml aliquots of blood from domestic cats, taken under anaesthesia, were collected into heparinised saline, washed in 0.9% saline, and then haemolysed with an equal volume of distilled water. Haemoglobin was extracted using chloroform/ethanol denaturation. The resulting supernatant solution, which contained largely carbonic anhydrase was dialysed overnight against tapwater and then buffered with 0.005 M Tris, pH 8.0. (b) Caecal mucosa and other tissues: mucosa was scraped from caecum with a spatula, and immediately frozen. Caecal mucosa was homogenised, using a glass Potter homogeniser. Cell debris was removed by centrifugation at 2000 × *g* for 10 min.

Liver and spleen samples were homogenised as described above. Small segments (approx. 1 g) were homogenised in about 2 ml of distilled water.

Results

Red cell carbonic anhydrases. In a preliminary screening of haemolysates from several members of the cat family (domestic cat, lion, jaguar, tiger and leopard), by electrophoresis, only one major band of carbonic anhydrase activity was found in all species tested. Location of esterolytic activity of carbonic anhydrase gave a strong fluorescent zone with fluorescein diacetate and a very weak zone with umbelliferone acetate reaction mixture, (this being typical for carbonic anhydrase II isozymes [2]).

The cat (*F. catus*) enzyme was studied in detail. All experimental procedures for cat red cell carbonic anhydrases were identical to those used in purification of the dog blood carbonic anhydrase isozymes [2]. The carbonic anhydrases from erythrocytes of the cat were finally purified by chromatography on DE-52 (Whatman). The partially purified extract was applied to a 50 ×

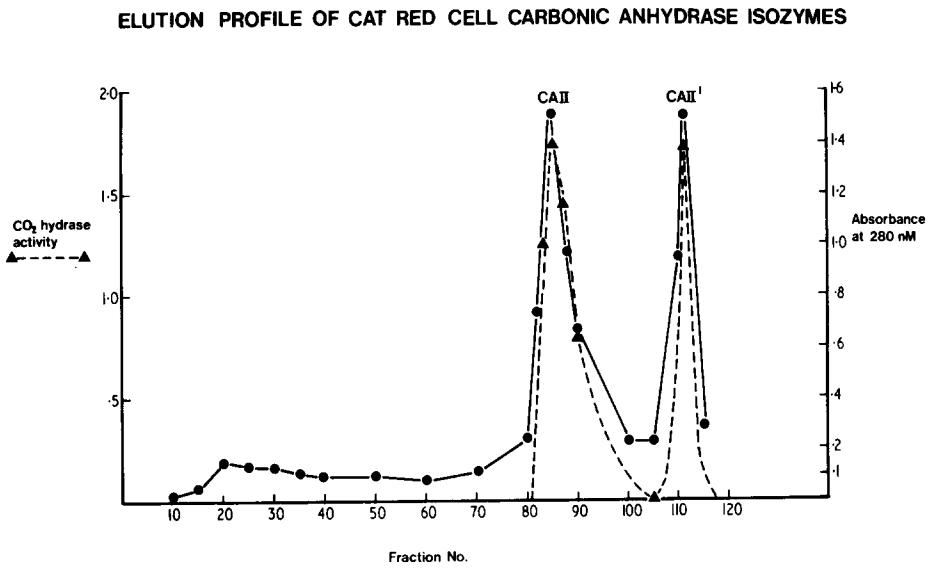

Fig. 1. Elution profile of crude feline carbonic anhydrase from DEAE-cellulose. The enzyme was eluted with an exponential gradient (500 ml 0.005 M Tris at pH 8.0 to 500 ml 0.2 M Tris, pH 8.0). Fraction volumes of 6 ml were collected.

2.5 cm column and eluted with a gradient of Tris buffer at pH 8.0. The protein and enzyme activity profile is shown in Fig. 1.

Two major protein peaks were observed, carbonic anhydrase II and carbonic anhydrase II[1], with activity profiles characteristic of the high activity

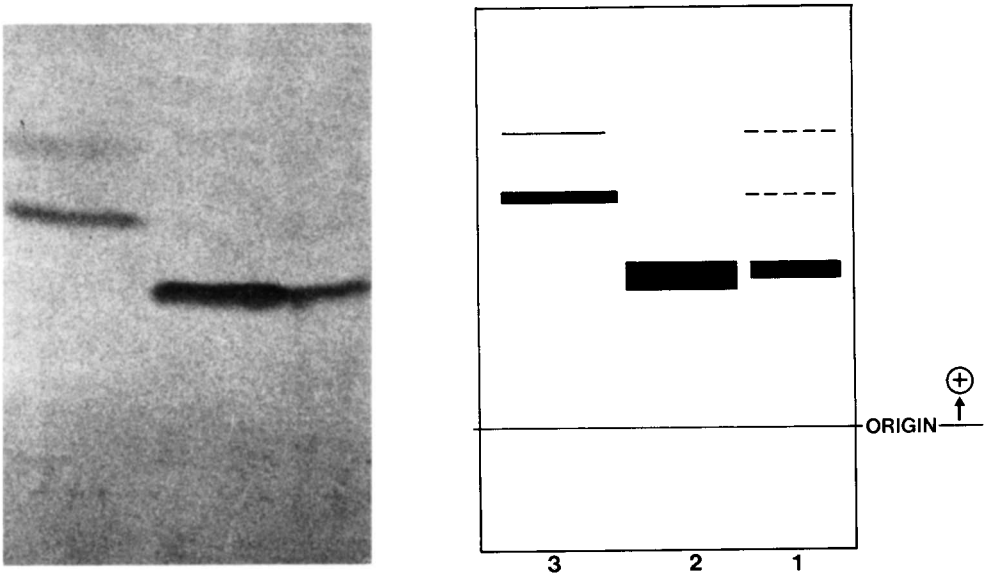

Fig. 2. Separation of cat carbonic anhydrase fractions by electrophoresis at pH 9.1. 1, crude extract before chromatography; 2, major carbonic anhydrase II peak; 3, carbonic anhydrase II[1] peak. The strip was stained with nigrosine.

isozyme form. The peak fractions were pooled separately, dialysed and lyophilised.

Electrophoresis of the crude red cell extract and the two purified carbonic anhydrase peaks after DE-52 chromatography and lyophilisation was carried out, and is illustrated in Fig. 2.

Caecal carbonic anhydrases. Electrophoresis of crude caecal homogenates gave a zymogram as shown in Fig. 3, (red cell lysate is also shown for comparison). Localisation of carbonic anhydrase zones, using the bromothymol blue-CO_2 activity method gave two zones of activity, one which corresponded to the red cell isozyme and another, considerably more basic in character which migrated towards the anode. Localisation of this more basic carbonic anhydrase using umbelliferone acetate [2] and specific reactions with carbonic anhydrase I antiserum (see below), indicated that this isozyme was of the carbonic anhydrase I (low activity) type. Examination of homogenates of cat liver and spleen also gave the zones of activity, one corresponding to red cell carbonic anhydrase II and the other zone to that found in cat caecum (carbonic anhydrase I). Because of the peculiarly basic nature of caecal carbonic anhydrase, and also the high concentration in mucosa, purification was relatively simple. Crude homogenates were dialysed against 0.005 M Tris at pH 9.0 and applied to a column 25 × 1.0 cm of DE-52 (DEAE-cellulose). Elution was carried out with 0.005 M Tris, pH 9.0. The carbonic anhydrase I isozyme eluted early in the experiment, was found to be homogeneous by electrophoresis on Cellogel.

Pooled material from seven animals was used to purify caecal carbonic anhydrase, and the resulting yield of several mg of enzyme was used for further characterisation.

Kinetic studies. The specific activities of purified red cell carbonic anhydrase II enzyme fractions and caecal carbonic anhydrase I were measured with CO_2, *p*-nitrophenyl acetate, fluorescein diacetate and umbelliferone ace-

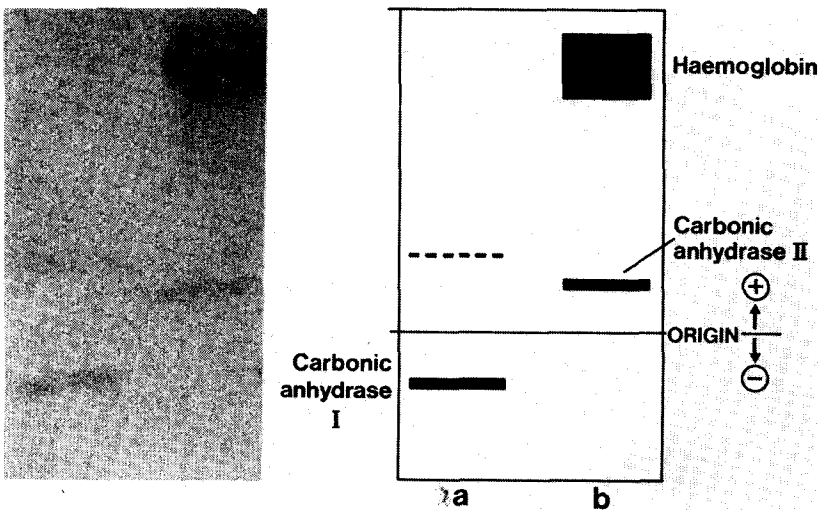

Fig. 3. Electrophoresis of a crude cat caecal homogenate and haemolysate Cellogel, with protein staining. Slot a, caecal homogenate; Slot b, crude haemolysate.

TABLE I

The specific activity is expressed as μmol/min per mg enzyme; n.d., not determined.

	CAT			DOG	
	Red cells		Caecum	Canine red cells	
	II	II[1]	I	I	II
Esterase activity with *p*-nitrophenyl acetate	1.55	1.21	0.079	0.081	0.77
CO_2 hydrase (Wilbur and Anderson [4], units/mg protein)	1830	1830	204	640	3160
Esterase activity with fluorescein diacetate	$0.35 \cdot 10^{-2}$	n.d.	0	0	$0.24 \cdot 10^{-2}$
Esterase activity with umbelliferone acetate	$0.009 \cdot 10^{-2}$	n.d.	$0.144 \cdot 10^{-2}$	0.115×10^{-2}	$0.046 \cdot 10^{-2}$

tate. The results are shown in Table I with the comparable results for the two dog carbonic anhydrase isozymes.

Reaction with antiserum. The purified red cell and caecal carbonic anhydrases from the domestic cat (*F. catus*) and haemo-lysates from jaguar, leopard, lion and tiger were tested with anti-human carbonic anhydrase I serum. Members of the dog family were tested in the same way and reacted with anti-human carbonic anhydrase I serum giving a strong precipitin line (Fig. 4). No reaction was found with any of the cat family red cell carbonic anhydrase; cat caecal homogenates gave a strong precipitin line with carbonic anhydrase I antiserum (Fig. 5).

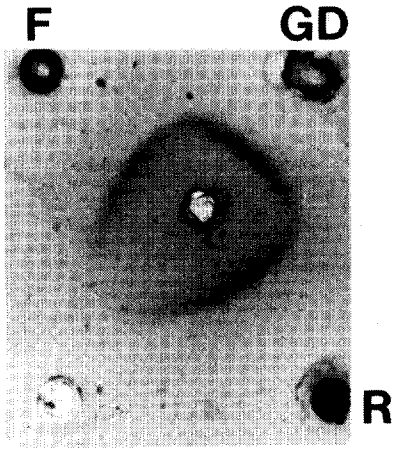

Fig. 4. Ouchterlony plate showing the reaction of dog family lysates: F-arctic fox, GD-greyhound and R-retriever against rabbit anti-human carbonic anhydrase I serum, (centre well).

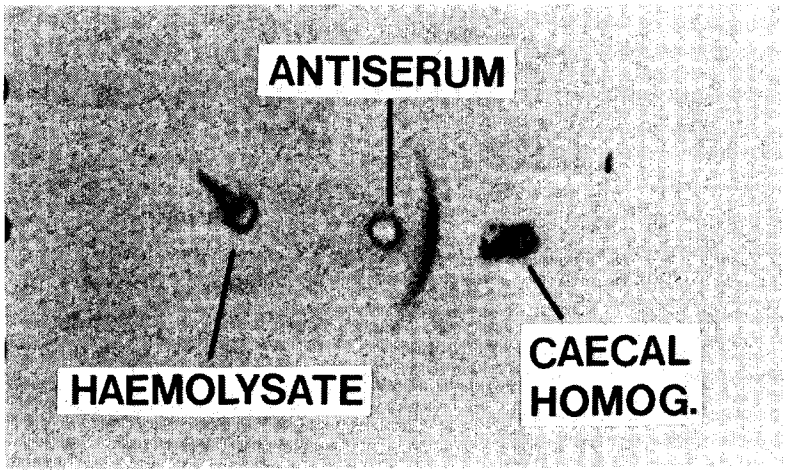

Fig. 5. Ouchterlony plate showing the reaction of cat caecal homogenate (right hand well), and cat haemolysate (left hand well) against rabbit, anti-human carbonic anhydrase I serum (centre well).

Amino acid analysis. Pooled results of amino acid analysis of both cat carbonic anhydrase II peaks and cat caecal carbonic anhydrase I are given in Table II; alongside these results is given the analysis of the dog isozymes, calculated using similar molecular parameters to those given for the cat enzyme [2].

TABLE II

The amino acid residue numbers in the present study have been "normalised" to a total molecular weight of 28 000, excluding tryptophan, which was measured separately. Serine and threonine were extrapolated to zero time of hydrolysis. Cysteine was measured as cysteic acid. Previous values for dog (based on a molecular weight of 28 000) are taken from ref. 2.

Amino acid	Cat carbonic anhydrase II		Dog carbonic anhydrase II*	Cat carbonic anhydrase I		Dog carbonic anhydrase I*
	Found	Nearest integer	Nearest integer	Found	Nearest integer	Nearest integer
Lysine	22.5	23	25	18.1	18	22
Histidine	11.1	11	12	11.5	12	10
Arginine	9.08	9	8	10.1	10	6
Aspartic acid	27.7	28	30	34.0	34	34
Threonine	12.9	13	17	11.0	11	11
Serine	18.6	19	13	25.0	25	24
Glutamic acid	24.7	25	29	23.9	24	25
Proline	19.5	20	21	21.8	22	21
Glycine	20.3	20	23	23.8	24	23
Alanine	17.1	17	18	20.5	21	20
Valine	14.7	15	16	16.9	17	18
Methionine	1.2	1	2	0.7	1.0	2
Isoleucine	14.7	15	15	15.4	15	18
Leucine	28.1	28	24	21.0	21	23
Tyrosine	8.4	8	6	7.1	7	5
Phenylalanine	14.1	14	12	10.2	10	9
Tryptophan	5.1	5	6	—	—	4
Cysteic acid	2	2	2	1.6	2	2

* Ref. 2.

Discussion

There has been some doubt as to whether carnivores possessed a low activity carbonic anhydrase I isozyme form and originally only a carbonic anhydrase II type isozyme in dog blood was reported [8,9]. Recent evidence, however, has shown two primary isozymes to be present in dog red cells [2]. A number of C-terminal amino acid sequences have been described from mammalian carbonic anhydrases [10] and the cat terminal residues were found to be characteristic of mammalian carbonic anhydrase II, whereas the dog isozyme which was purified exhibited a carbonic anhydrase I-type terminal sequence. From the present study it is clear that members of the dog family have red cell carbonic anhydrase I and carbonic anhydrase II isozymes, whereas the cat family has only one genetically distinct form of carbonic anhydrase II in red cells.

The evidence for the separate identity of cat carbonic anhydrase I (caecum) and carbonic anhydrase II (red cells) in this study, relates to (a) amino acid analysis, (b) differential esterase specificity for fluorescein diacetate and umbelliferone acetate as a substrate [2], (c) other kinetic characteristics as shown in Table I, (d) reaction with antiserum. As far as cat red cell carbonic anhydrase isozymes are concerned, the characteristics found were typical of most other mammalian carbonic anhydrase II. Two red cell isozymic forms were purified during this study, a major component and an anodal "satellite" (carbonic anhydrase II^1 in Fig. 1).

The occurrence of "satellite" or modification components after electrophoresis of mammalian carbonic anhydrases is common. In the case of human enzyme, ageing of enzyme preparations results in progressive loss of amide groups, with concomitant increase in carboxylic acid groups and thus increased anodal migration during electrophoresis [11]. In the cat the amino acid analysis of peaks of red cell carbonic anhydrase II and carbonic anhydrase II^1 were identical within the limits of experimental error, and the pooled analysis is shown in Table II, (amide groups were not measured in this study). Comparison with dog carbonic anhydrase II shows a marked similarity and the serine levels, in particular, are characteristic of mammalian carbonic anhydrase II isozymes. The formation of carbonic anhydrase II^1 (see Fig. 2) seems likely to be due to an analogous deamidation process to that found with human carbonic anhydrase on ageing in vitro. Additional evidence for this is that the carbonic anhydrase II peak, after chromatography, lyophilisation and storage at 4° C, progressively developed small amounts of a component identical in electrophoretic migration to carbonic anhydrase II^1. An electrophoretic survey of lysates from several cats revealed identical phenotypes to that preparation used for characterisation, showing that genetic heterogeneity was not causing multiple isozymes in this particular case.

Immunological evidence, using carbonic anhydrase I antiserum, showed that whereas several members of the dog family have marked expression of a carbonic anhydrase I immuno-reactive protein in red cells (Fig. 4) this was not observed in red cells of members of the cat family. However, detailed qualitative and quantitative examination of homogenates of caecal mucosa from the domestic cat showed that a high level of a carbonic anhydrase I type isozyme was present (Fig. 3). This carbonic anhydrase I was immunologically and kineti-

cally very similar to dog red cell carbonic anhydrase I, but was found to be more basic in electrophoretic characteristics (see Fig. 3). Amino acid analysis showed (Table II), that cat caecum carbonic anhydrase I has a composition similar to other mammalian carbonic anhydrase I isozymes, with a characteristically high level of serine compared to carbonic anhydrase II. Spleen and liver homogenates of the cat also contained carbonic anhydrase I isozyme, judged by electrophoresis and staining with bromothymol blue.

The findings for caecal carbonic anhydrase I in the cat are very similar to those for ox rumen [12] where carbonic anhydrase I was found to be absent from red cells, but expressed in the rumen. In the ox, the rumen carbonic anhydrase I showed an unusually high content of basic amino acids, with a ratio of basic amino acids carbonic anhydrase I/carbonic anhydrase II of 1.0, whereas for other mammalian carbonic anhydrases this ratio ranged from 0.81 to 0.85. In the case of the cat the ratio of basic amino acids was also close to 1.0 (Table II). It seems possible that this may be of some functional advantage, for example in providing a charge interaction to enable binding to subcellular sites.

Further information on the mechanism of tissue carbonic anhydrase induction may be forthcoming from a study of a regulatory variant of carbonic anhydrase I recently described in the chinchilla (*Chinchilla laniger* [13]). Measurement of carbonic anhydrase I in the caecum of chinchilla with an inherited, almost total, red cell deficiency showed that there was a normal level of carbonic anhydrase in the caecum of these animals. The genetic analysis of this variation shows that animals homozygous for the deficiency (carbonic anhydrase I (—)) contain less than 1% of red cell carbonic anhydrase I compared with carbonic anhydrase I (+) animals. Assay of red cell carbonic anhydrase I in chinchillas of carbonic anhydrase (+—) heterozygous phenotypes showed 50% levels of carbonic anhydrase I. This variation of carbonic anhydrase I appears to result from a mutation in a regulatory gene, since the qualitative structure of carbonic anhydrase I in the different quantitative phenotypes is apparently unchanged in the deficient animals. An extension of this model to the cat and ruminants would be that the lack of carbonic anhydrase I in these latter species may result from fixation of a gene for carbonic anhydrase I repression in red cells; no cats or ruminants with carbonic anhydrase I production in red cells have been found. The exact mechanism of specific repression of carbonic anhydrase I synthesis in red cells remains to be elucidated, and may provide a clue to the mechanisms of control of protein biosynthesis in mammals.

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