EVIDENCE FOR HIGH AND LOW ACTIVITY CARBONIC ANHYDRASES IN THE RED CELLS OF THE DOG

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1. Introduction

In most mammalian species carbonic anhydrase (EC 4.2.1.1.) exhibits an unusual type of polymorphism due to the presence in erythrocytes of two genetically distinct isozymes which differ strongly in specific activity, amino acid composition and immunological properties. In this paper they will be designated by CAI for the low-activity form and CAII for the high-activity form*.

Some exceptions have been noted. They first concern some Ruminants in the red cells of which only CAII has been found [1–5]. Absence of carbonic anhydrase of low activity has been also pointed out in the erythrocytes of the carnivores, dog and cat. Concerning the dog, the results differ according to the authors. All of them [6,7,9] but N. D. Carter [8] have isolated only one form of carbonic anhydrase; however from their results it is not clear whether it is a CAI or a CAII isozyme.

We undertook to elucidate this problem using of affinity chromatography, which was adapted to the isolation of carbonic anhydrases in 1972 [10], it allows separation of the two isozymes on the basis of the differences in their affinity for sulfonamides and monovalent ion inhibitors. In the method recently worked out in our laboratory, after adsorption on CH-Sepharose 4B coupled with sulfanilamide, CAI forms were specifically eluted by NaI and CAII forms by KCNO [11]. By application of this technique we were able to isolate in pure form the two isozymes CAI and CAII from the dog red cells.

* In order to unify the nomenclature. CAI and CAII were previously referred respectively B and C by us, as by several authors.

2. Materials and methods

After centrifugation of the heparinized blood the red cells were washed with 0.9% NaCl and hemolysed by two volumes of water. Crude extract of carbonic anhydrase was prepared by filtration of the hemolysate on Sephadex G-75 according to Rickli et al. [12]. Isolation of carbonic anhydrases CAI and CAII was performed by affinity chromatography as described by Marriq et al. [11]. Protein concentration was determined by the Lowry technique [13]. Carbonic anhydrase activity was determined by the Wilbur–Anderson method [14]. The enzyme units (EU) were calculated according to the formula: $EU = (t_0/t) - 1$, where t_0 is the time of the uncatalyzed reaction and t the time of the enzyme-catalyzed reaction. Amino acid analyses were performed with a Beckman Multichrom analyzer. Tryptophan was obtained after hydrolysis of the protein in the presence of thioglycolic acid [15] and cystein after performic oxydation [16]. Number of residues was calculated on the basis of a mol. wt. of 30 000. Immunological reactions were carried out by the technique of Ouchterlony [17]. Further details of the experimental procedures will be described elsewhere [18].

3. Results

Fig. 1 reports the elution pattern obtained by filtration of the hemolysate on Sephadex G-75.

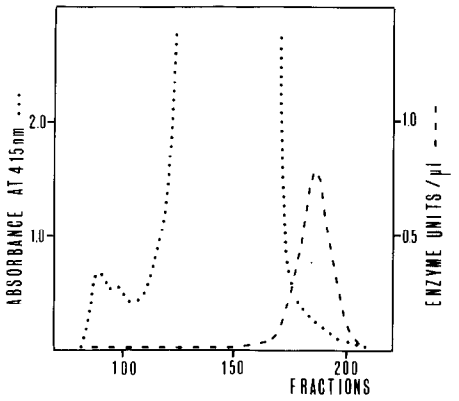

Fig.1. Filtration on Sephadex G-75 of hemolysate from 10 ml of dog erythrocytes. Equilibration and elution with 0.1 M Tris sulphate, pH 7.5. Column dimensions: 5 × 100 cm. Flow rate: 50 ml/h. Fractions size: 5 ml.

Ninety-nine per cent of hemoglobin is eliminated with a recovery of 65% of the initial enzymic activity.

Fig.2 shows the results of affinity chromatography:

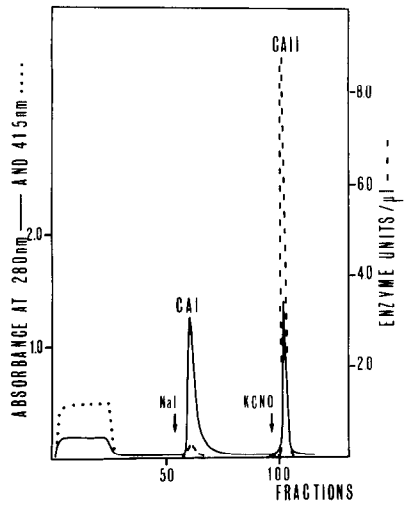

Fig.2. Affinity chromatography of crude extract of erythrocyte carbonic anhydrase of dog. Active fraction from the Sephadex column corresponding to 50 mg of total protein was directly applied to the substituted Sepharose column (0.9 × 14 cm) equilibrated in 0.1 M Tris-sulphate, pH 7.5. Non-specifically bound proteins were eluted with the equilibration buffer. CAI form was eluted with 0.1 M Tris-sulphate–0.1 M NaI, pH 7.5, and CAII form with 0.1 M Tris-sulphate–0.02 M KCNO, pH 6.6. Flow rate: 20 ml/h. Fractions size: 7 ml until fraction 50, then 2 ml.

Table 1
Activity of erythrocyte carbonic anhydrases of dog and some other mammals

		Enzyme units/mg
CAI	Dog	1080
	Man	1560
	Rat	1100
CAII	Dog	15 000
	Man	11 400
	Rat	15 700
	Ox	20 600

two active fractions were obtained, the first one of low activity (CAI) eluted by NaI and the second one of high activity (CAII) eluted by KCNO. The relative proportions of the two isozymes were about 60% of CAI and 40% of CAII.

After dialysis enzymic activity was determined. The results are reported in table 1 together with the values obtained in the same experimental conditions

Table 2
Amino acid composition of erythrocyte carbonic anhydrases of dog

Amino acid	Carbonic anhydrases isolated by: CAI	CAII	Byvoet	Soliman
	Number of residues per molecule			
Asp	35.1	29.1	33.6	35.2
Thr	10.2	12.2	11.2	13.6
Ser	31.7	18.0	24.9	28.2
Glu	22.7	27.8	26.3	25.0
Pro	17.6	17.9	19.0	17.6
Gly	17.5	22.3	24.4	20.2
Ala	17.1	17.4	17.5	16.5
Val	15.3	13.0	14.8	14.9
Cys	1.1	2.2	1.2	2.0
Met	1.1	2.2	–	1.9
Ile	17.0	12.0	12.7	14.7
Leu	21.7	25.3	24.5	22.3
Tyr	12.4	8.0	7.8	9.3
Phe	9.5	13.0	11.5	9.3
Lys	19.4	22.9	22.3	20.2
His	12.2	13.4	11.9	10.9
Arg	6.3	7.2	6.9	7.9
Trp	6.1	6.5	6.4	5.8
Total	274.0	270.4	276.9	275.5

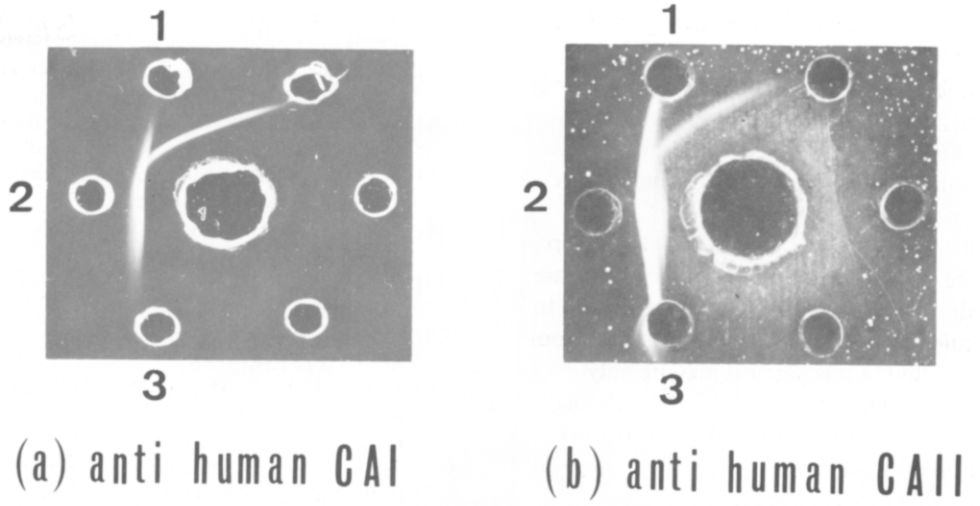

Fig.3. Ouchterlony double diffusion tests of dog carbonic anhydrases against rabbit antisera of human CAI and CAII. (a) Central well was filled with 60 μl of human CAI antiserum Peripheral wells were filled with (1) 5 μg of dog CAI; (2) 5 μg of human CAI; (3) 5 μg of dog CAII. (b) Central well was filled with 200 μl of human CAII antiserum. Peripheral wells were filled with (1) 5 μg of dog CAII; (2) 10 μg of human CAII; (3) 5 μg of dog CAI. The (b) plate was stained with amido black.

for some mammalian erythrocyte carbonic anhydrases previously isolated in our laboratory by affinity chromatography [11,18].

Table 2 lists the amino acid composition of CAI and CAII. For comparison the data concerning the single form isolated by Byvoet et al. [6] and by Soliman et al. [7] have been included. Very significative differences exist between the two isozymes. It will be particularly noted that the high content of serine of CAI is consistent with those generally observed in the low activity forms of carbonic anhydrases.

Immunological reactions were carried out against rabbit antisera specific for the human red cells CAI and CAII. Cross reaction was only observed between the homologous enzymes (fig.3). But, if dog CAI reacts strongly with anti-human CAI the precipitation between dog CAII and anti-human CAII is only visible after coloration with amido black.

In some experiments hemoglobin was eliminated by chloroform-ethanol denaturation. Recovery of activity was lower than that obtained after Sephadex filtration but no significative difference was observed between the isozymes isolated from the two types of crude extract.

4. Discussion

From the present study it is clear that the erythrocytes of dog contain two forms of carbonic anhydrase which have respectively the characteristics of CAI and CAII. They are eluted from Sepharose in the same conditions as the homologous enzymes of human [11] and rat [18] red cells. Their specific activities, differing by a factor nearly 15, is of the same order as those of the other CAI and CAII isozymes studied (table 1). From their immunological behaviour it is also evident that they respectively belong to the low and high activity forms. In contrast to the results obtained by N. D. Carter [8] we did not observe any cross reaction between the dog CAII and the human CAI antiserum.

The difficulties previously encountered in separating the two isozymes of carbonic anhydrase from the erythrocytes of dog [6,7] are easily understandable. Their electrophoretic mobilities at pH 8.2 are very close and we also failed to separate them by chromatography on ion exchanger. Only one active fraction was then obtained. Its activity is intermediate between that of CAI and CAII and corresponds to that given by Soliman [7] for the unique form isolated.

It should however be noted that the presence of CAI in dog red cells could be presumable from previous immunological results [19] showing the strong cross reactivity between dog hemolysate and anti-human CAI. On the other hand this antiserum did not react against cat hemolysate in agreement with the results obtained by Carter [8].

The number of carnivores tested is not sufficient to know if the cat and probably the other felidae are together with the ruminants the unique exception to the general rule of polymorphism in mammals. From the results reported in this paper it may be only concluded that absence in the red cells of carbonic anhydrase of low activity is not a common feature of all the carnivores.

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