A NOVEL CARBONIC ANHYDRASE FROM THE OVINE PAROTID GLAND

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1. Introduction

The parotid gland of the sheep is capable of producing large amounts of bicarbonate which is secreted into the saliva at rates as high as 50 mmol . h^{-1} [1]. This suggests that there is either an active carbonic anhydrase system in the gland or an efficient bicarbonate transfer from blood to saliva (or a combination of the two processes).

As part of a study on the physiology of bicarbonate secretion [2], we wished to quantitate the parotid carbonic anhydrase activity, which is known to be high in some species [3]. In the course of these investigations we observed a high molecular weight form of carbonic anhydrase that is clearly different from the commonly studied forms, as exemplified by the erythrocyte enzymes, which are widely distributed in mammalian tissues.

2. Experimental

2.1. *Enzyme preparation*

Parotid, kidney, stomach, brain and liver were obtained from merino sheep following perfusion of the anaesthesized animal with cold normal saline to remove blood cells from the tissue. The tissues were homogenized at 4°C in a Waring blender in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.5 mM phenylmethylsulfonylfluoride. Following centrifugation (20 000 × *g* for 20 min) the supernatants were applied to a 90 × 2.5 cm column of Sephadex G-75 superfine (Pharmacia) in 0.1 M NH_4HCO_3 (pH 8.0). Ovine erythrocyte carbonic anhydrase was prepared by the method in [4]. Saliva was collected from sheep by cannulation of the parotid duct, then concentrated by ultrafiltration before being applied to the Sephadex G-75 column.

2.2. *Enzyme assay*

The carbon dioxide hydrase activity was measured by the velocity of the change in pH from 7.8 to 7.5 in a buffered solution at 20°C through which CO_2 was bubbled.

2.3. *Molecular weights*

The method in [5] was used to measure the molecular weights of the enzymes. Columns of Sephadex G-75 and Sepharose 4B were used with ribonuclease and bovine erythrocyte carbonic anhydrase (Boehringer Mannheim) and pepsin (Sigma) as markers for the first column and ovine gamma globulin, urease (Calbiochem) and red blood cell catalase as markers for the second column.

2.4. *Antibody formation*

Antibodies against the ovine erythrocyte carbonic anhydrase were raised in rabbits by injecting an emulsion of the purified enzyme in Freund's adjuvant (Difco) at monthly intervals.

2.5. *Haemoglobin content*

Contamination of the parotid homogenate by erythrocyte haemoglobin was measured by the method in [6].

2.6. *Differential centrifugation*

Parotid tissue was homogenized in a Teflon and

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glass homogenizer and subjected to differential centrifugation as in [7]. Precipitates were washed 3 times with buffer, were incubated at 37°C for 30 min in 0.5% Triton X-100 in 0.1 M Tris–HCl (pH 7.5) then centrifuged. Suspensions of these precipitates were assayed for enzyme activity.

3. Results

3.1. *Tissue carbonic anhydrase*

The ovine kidney, brain, liver and stomach homogenates, chromatographed on the Sephadex G-75 columns showed a single peak of carbonic anhydrase activity with K_{av} values consistent with mol. wt ~30 000. A profile of the kidney homogenate on Sephadex G-75, typical of the 4 tissue runs, is shown in fig.1. These partially purified preparations were used to measure the degree of inhibition by azetazolamide and 2-sulfonamido-1,3,4-thiadiazole-5-benzene sulfonamide (CL11366), the specific and powerful sulfonamide inhibitors of carbonic anhydrase. The values obtained for the tissue enzymes were similar to that of the erythrocyte enzyme and to other reported values [3]. The renal enzyme was found to be inhibited to the same extent as erythrocyte carbonic anhydrase by antibodies raised against the erythrocyte enzyme (table 1).

3.2. *Parotid carbonic anhydrase*

When parotid homogenate was chromatographed on Sephadex G-75, two peaks of carbonic anhydrase activity were observed (fig.2). Concentrated saliva showed a similar activity profile when run on the

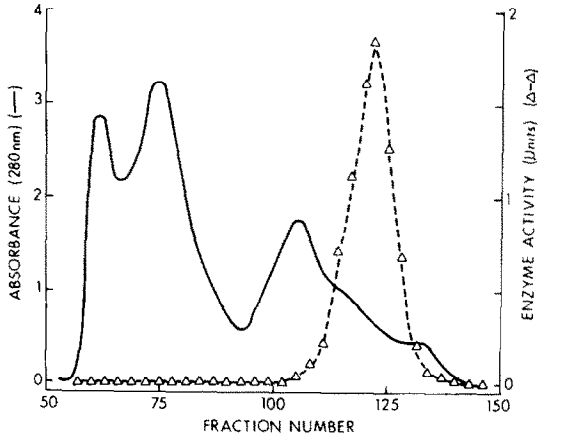

Fig.1. Elution profile of carbonic anhydrase activity (△) of ovine kidney homogenate on a column of Sephadex G-75 superfine (90 × 2.5 cm) equilibrated in 0.1 M NH_4HCO_3 buffer (pH 8.0).

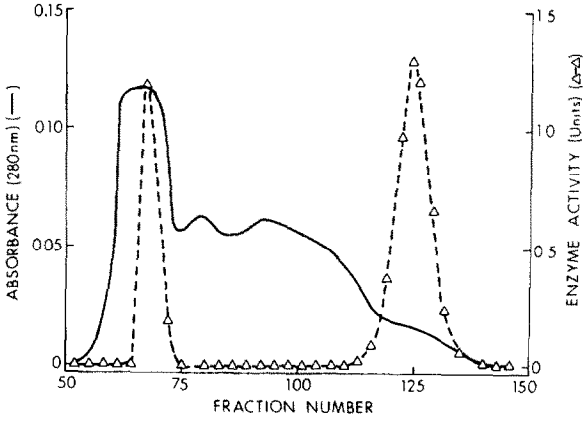

Fig.2. Elution profile of carbonic anhydrase activity (△) of parotid homogenate on a column of Sephadex G-75 superfine (90 × 2.5 cm) equilibrated in 0.1 M NH_4HCO_3 buffer (pH 8.0).

Table 1
Molecular weights and inhibition properties of carbonic anhydrases isolated from various tissues of the sheep

Tissue	Mol. wt (approx.)	Acet.[a] I_{50} (nM)	CL11366 I_{50} (nM)	Inhibition by 1 mM NaN_3 (%)	Inhibition by antibody (%)
Erythrocyte	30 000	43	16	39	78
Kidney	30 000	43	16	37	78
Brain	30 000	59	20	b	b
Liver	30 000	43	16	b	b
Stomach	30 000	38	11	b	b
Parotid – peak II	30 000	47	11	39	66
Parotid – peak I	238 000	100	100	46	~6

[a] Acetazolamide; [b] value not determined

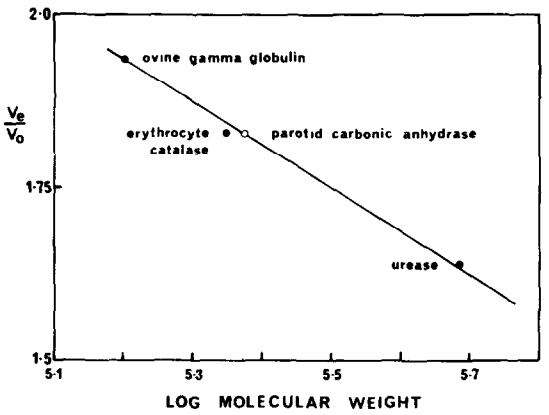

Fig.3. Molecular weight estimation of ovine parotid carbonic anhydrase. Proteins of known molecular weight (ovine gamma globulin, erythrocyte catalase and urease) were chromatographed on a column of Sepharose 4B in 0.1 M NH_4HCO_3 buffer. The ratios of their elution volumes to the void volume were plotted against the log of their molecular weights. The position of the parotid carbonic anhydrase is indicated by the circle.

same column. One peak (II) emerged in the expected position (V_e/V_o 1.35), but another peak (I) was found closer to the void volume. Peak II appears very similar to the erythrocyte enzyme in its molecular weight, sulfonamide inhibition and inhibition by antibody (table 1).

The larger carbonic anhydrase had app. mol. wt 238 000 by gel filtration on Sephadex 4B (fig.3). The enzyme remained as a single high molecular weight peak on rechromatography in 0.1 M ammonium bicarbonate, in 2 M NaCl or in 0.5% Triton X-100. The activity was not sensitive to chloride inhibition (I_{50} 950 mM) and showed similar susceptibility to inhibition by azide as the erythrocyte enzyme. However, this enzyme is less susceptible to sulfonamide inhibition than the other enzymes and is not inhibited by the antibody raised against the erythrocyte carbonic anhydrase (table 1).

3.3. *Haemoglobin content*

The concentration of haemoglobin measured in the parotid homogenate indicated that <3% of the total carbonic anhydrase activity of the homogenate was derived from contaminating erythrocytes.

3.4. *Differential centrifugation*

Most of the carbonic anhydrase activity remained in the 100 000 × *g* supernatant on differential centrifugation with very little activity in the washed precipitates. Incubation of these precipitates with 0.5% Triton X-100 did not solubilize appreciable amounts of enzyme activity. The activity profile for 100 000 × *g* supernatant on the Sephadex G-75 column was the same as for the parotid homogenate as described above.

4. Discussion

A different type of carbonic anhydrase has been found in homogenates of the ovine parotid gland and in the saliva. Whereas all vertebrate carbonic anhydrases previously reported are monomers with mol. wt ~30 000 [8], this enzyme has mol. wt 238 000 and shows no immunological crossreactivity with antibodies to the erythrocyte enzyme. While it has been shown that sheep erythrocytes contain only one major form of carbonic anhydrases [9], the C or high activity isoenzyme, the less active B isoenzyme has been found in other tissues of this species [10]. Although the B isoenzyme would probably not crossreact with antibodies raised against the erythrocyte (C) enzyme [11] it is nevertheless distinct from the large parotid enzyme.

Both enzymes (peaks I, II) are endogenous to the parotid gland and are not from contaminating erythrocytes. The differential centrifugation and gel filtration studies suggest the parotid enzyme is of cytoplasmic origin and is not an aggregate of the small carbonic anhydrase and other proteins or membrane fragments. No significant CO_2 hydrase activity was associated with any particulate fraction although such membrane-bound activity has been found in other tissues [12,13].

Of the ovine tissues studied, the parotid was the only one found to contain this high molecular weight carbonic anhydrase and in this gland it constituted 40–50% of the total CO_2 hydrase activity. By size and inhibition criteria, the small enzyme appears to be identical with the erythrocyte carbonic anhydrase. The role of the 2 enzymes in bicarbonate secretion in the salivary gland is as yet not clear, however, taking into account the great variability in bicarbonate output and the large size and probable subunit composi-

tion of the peak I carbonic anhydrase, the possibility of some form of control on the activity of the enzyme exists.

Work is in progress on the purification and characterization of this unusual carbonic anhydrase.

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