THE ISOLATION OF CARBONIC ANHYDRASE FROM THE MUSCLE OF *SQUALUS ACANTHIAS* AND *SCOMBER SCOMBRUS*: INHIBITION STUDIES.

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Phylogenetic studies have revealed the occurrence of carbonic anhydrase (CA) in the red cells of vertebrates as primitive as the agantha (Carlsson et al. Biochim. Biophys. Acta 612,160,1980) and the elasmobranch (Maynard and Coleman, J. Biol. Chem. 246,4455,1971). Isozymic evolution giving rise to the largely homologous but distinctly different red cell isozymes I and II started at least as early as in the reptilian era (Hall and Shraer, Comp. Biochem. Physiol. 63B,561,1979). A third genetically distinct isozyme, CA III, occurs in the skeletal muscle of mammals and avians (Holmes, Eur. J. Biochem. 78,511,1977). We have now extended the phylogenetic study on muscle CA and found CA activity in the red muscle of dogfish, an elasmobranch, and mackerel, a teleost. The dogfish and mackerel muscle enzymes have been isolated, free of erythrocyte CA contamination, by a two-stage affinity chromatography method described by Sanyal et al. (Mol. Pharmacol. 22,211,1982). We discuss here some active site characteristics of these enzymes with emphasis on the effects of thiadiazole sulfonamides and inorganic anions, and compare these enzymes with mammalian CA isozymes I, II and III.

Methods. Red muscle was dissected from heparinized and freshly sacrificed e shark or mackerel. The tissue was homogenized in a hand-held tissue grinder using 0.05 M Tris buffer at pH 7.5 containing 0.25 M K_2SO_4 and 1 mM EDTA. The two affinity resins were freshly prepared by coupling CM-Biogel A (Bio-Rad) with (i) para-aminomethylbenzenesulfonamide (PAMBS, from Sigma) (ii) and para-aminobenzolamide (CL 13475, from Lederle). The complete removal of contaminating red cell CA activity by the PAMBS affinity resin was confirmed by the inability of methazolamide (Lederle), even at 10 µM, to inhibit the remaining CA activity in the PAMBS gel treated muscle supernatant. This supernatant was then loaded on the CL 13475 affinity gel. The gel was extensively washed with 0.05 M Tris buffer at pH 7.5 also containing 0.25 M Na_2SO_4. Elution of protein containing CA activity was achieved by inclusion of 0.4 M NaN_3 in the same buffer at pH 7.5. The pooled fractions were concentrated by ultrafiltration and dialyzed against 5 mM Tris buffer containing 30 mM Na_2SO_4 at pH 7.1. Dialysis against water alone appeared to cause partial loss of protein by precipitation.

The CO_2 hydration assays were done at 1°C according to Maren et al. (J. Pharmacol. Exp. Ther. 130,389,1960) using non-inhibitory barbital buffer in a total reaction volume of 1 ml. Zinc determination was carried out by atomic absorption spectrometry. Determinations of protein concentrations were carried out by the method of Lowry et al. (J. Biol. Chem. 193,265,1951) using bovine CA II (Sigma) as the reference. Fluorescence experiments were carried out using a SLM 4800 phase fluorometer (SLM-Aminco, Urbana-Champaign, Illinois). Tryptophan fluorescence was excited at 280 NM (bandpass = 2 NM) and emission was observed from 300 to 450 NM (bandpass = 4 NM).

RESULTS AND DISCUSSION

Inhibition. The critical data on the inhibition of dogfish and mackerel muscle CA by sulfonamides and anions are shown in Table I. For comparison, our earlier data on mammalian red cell CA isozymes I and II, and muscle isozyme III are also given. The elasmobranch and the teleost muscle CAs align themselves with mammalian CA III in being highly refractory to sulfonamides relative to CA I and CA II (10^2 - 10^5 fold). Among the three sulfonamides we tested benzolamide stands out in being even 40 - 50 times less inhibitory against dogfish and mackerel muscle CAs compared to mammalian muscle CA III. An astounding difference between CA III and the elasmobranch and teleost CAs is, however, found when inhibition by anions is compared. Compared to CA III, dogfish and mackerel muscle CAs show about a 100-fold resistance against cyanate and iodide. These primitive muscle enzymes resemble CA II rather than CA III (or CA I) in this respect.

TABLE I

Inhibition by sulfonamides and anions of skeletal muscle CAs from dogfish and mackerel: comparison with mammalian CA - I, II and III.

Inhibitor	K_i (μM)				
	Dogfish[a] muscle	Mackerel[a] muscle	CA I[b] (red cell)	CA II[b] (red cell)	CA III[c] (muscle)
Methazolamide	70	65	0.01	0.01	96
Benzolamide	150	120	0.001	0.001	3
Chlorzolamide	0.4[d]	0.05[d]	0.001	0.0004	0.3
Cyanate	30	70	0.70	20	0.5
Iodide	90,000	--	300	26,000	1100

[a]Either the PAMBS gel-treated muscle supernatant or the more purified preparation eluted off the CL 13475 affinity column was used. Identical results were obtained when the two preparations were compared for inhibition by methazolamide and benzolamide.

[b]Anion inhibition data are taken from Maren et al. Science, 191,469,1966. Data for sulfonamides are taken from Sanyal et al. Biochim. Biochim. Acta 657,128,1981.

[c]Data of Sanyal et al. Mol. Pharmacol. 22,211,1982.

[d]I_{50} data were not corrected for enzyme concentrations since the relationship between EU and concentration has not been established. For dogfish 0.8 EU and for mackerel 0.3 EU were used.

Activity. By comparing the total protein concentration of the dogfish muscle CA preparation (obtained by double affinity chromatography) with the CO_2 hydrase activity, we have arrived at a rough value of 1×10^{-5} M for enzyme concentration in one EU for the 7 ml reaction system as defined earlier (Maren et al., J. Pharmacol. Exp. Ther. 130,389,1960). This is probably an underestimation of activity in view of the uncertainties in molecular weight (MW), presence of contaminant proteins and instability of the activity. For comparison, the values for CA-I, II and III are respectively 7×10^{-9} M, 1×10^{-9} M and 5×10^{-7} M (Maren and Couto, Arch. Biochem. Biophys. 196,501,1979; Sanyal et al., Mol. Pharmacol. 22,211,1982). The higher the concentration of enzyme required to generate one EU, the lower is the enzyme activity. Thus the elasmobranch muscle CA appears to be a low activity CA. Among the mammalian isozymes also, muscle CA III has the lowest activity. The activity of dogfish muscle CA exhibited a 70% decrease upon 4 hours of incubation with 1 mM pyridine-2,6-dicarboxylate at pH 6, suggesting zinc dependence of the activity. The native enzyme preparations were found to contain 1.1 - 1.4 equivalents of zinc.

In calculating the molar enzyme concentration in these and other experiments, a MW of 36,000 for dogfish CA was used. This number was obtained from SDS-polyacrylamide gel electrophoresis experiments which also showed some high MW bands (60,000 and above) and a low MW band (25,000). Aggregation, adsorption of contaminant proteins on the affinity gel and proteolytic cleavage are possible explanations.

Sulfonamide Binding. The binding of 2-chlorophenyl-1,3,4-thiadiazole-5-sulfonamide (chlorzolamide) to dogfish muscle CA was accompanied by a quenching of tryptophan fluorescence emission from the enzyme. Half-maximal quenching was observed at 5 μM chlorzolamide when the enzyme concentration was approximately 7 μM. This is consistent with chlorzolamide inhibition of activity (Table I). The fluorescence quenching effect of chlorzolamide disappeared when zinc was removed from the enzyme by extensive dialysis against pyridine-2,6-dicarboxylate at neutral pH.

CONCLUSIONS

1) The skeletal muscles of S. acanthias and S. scrombus contain CA. The evolutionary history of muscle CA is therefore at least as old as that of red cell CA-I and II.

2) The elasmobranch muscle CA is a low activity CA (mammalian muscle CA III is also a low activity enzyme compared to red cell CA-I and II) and, like all CAs known to date, its activity is zinc dependent.

3) Like CA III of mammalian muscle, CAs from elasmobranch and teleost muscle are highly refractory to sulfonamides (relative to CA-I and II).

4) The elasmobranch and the teleost muscle CAs are, however, different from mammalian CA III in being resistant to inhibition by anions. They resemble CA II in this respect.