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A SEARCH FOR THE FUNCTION OF HUMAN CARBONIC ANHYDRASE B

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Summary

Because of the very high activity and abundance of human red cell carbonic anhydrase C (carbamate hydrolase, EC 4.2.1.1), it seemed likely that the second isozyme, B, might not be essential for CO_2 metabolism. It was then found that physiological concentrations of Cl^- inhibited catalysis of CO_2 hydration by the B enzyme (but not by type C), suggesting further that type B does not function in vivo as a carbonic anhydrase. The versatility of the catalytic activity of carbonic anhydrase for a number of 'artificial' substrates suggested that enzyme B may be utilized in reactions of intermediary metabolism. A number of hydration, dehydration, decarboxylation, kinase, and phosphatase systems were tested to determine a possible physiological function for the enzyme. Results with eighteen possible substrates were negative and the possibility is discussed that mammalian carbonic anhydrase B is an evolutionary accident.

Introduction

Human red cells contain two zinc enzymes which catalyze the reversible hydration of CO_2 and the hydrolysis of certain esters [1]. Although the enzymes are of similar size and shape, closely related amino acid sequence and active site, they can be separated from blood on the basis of different electrophoretic properties; they have been designated as carbonic anhydrates (carbamate hydrolase, EC 4.1.1.1) B and C or I and II. A number of chemical differences enzymes between B and C which have been cited in the literature, collectively suggest that there may be different physiological roles for the two enzymes.

Among the chemical differences between carbonic anhydrase B and C are: a) The concentration of the B enzyme is 4 g (133 μM) per litre of red cells, and type C is 0.7 g (23 μM) per litre [2]; b) The turnover number of type C for CO_2 or HCO_3^- at pH 7.4 is 20 times greater than that for type B [2]; c) The

concentration of acetazolamine required to inhibit enzyme B is about 10 times that which is required to inhibit type C [2]; d) The sensitivity of carbonic anhydrase B to anion inhibition is up to 87 times the sensitivity of the C enzyme [3].

The most important evidence that enzyme B does not function in vivo as a carbonic anhydrase is the report that this enzyme is 93% inhibited at the usual Cl^- concentration in red cells (about 85 μM); carbonic anhydrase C is highly effective for CO_2 hydration in the presence of Cl^- [3]. In addition, studies of red cell physiology in respiration, clearly show that carbonic anhydrase C is entirely adequate for the respiration, even in situations of severe exercise [4].

Results and Discussion

It was the purpose of the present study to see if the physiological role of carbonic anhydrase B might be that of a catalyst for substrate(s) other than CO_2. During the past decade, various workers have shown the carbonic anhydrases possess catalytic activities for a number of nonphysiological substrates. Among the activities studied were the hydrolysis of various esters [5—10], the reversible hydration of a variety of aldehydes [11—13], and the enzymatic cleavage of 2-hydroxy-5-nitro-α-toluenesulfonic acid sulfone [14]. These reports of the versatility of the enzyme suggest that a physiological substrate may exist to explain the catalytic function of carbonic anhydrase B in primate red cells.

We were guided in our selection of possible compounds by the structural characteristics of the 'artificial' substrates and by the similarity of the catalytic mechanisms to physiological systems. In each case, optimal conditions for the normal enzyme activity were selected and three types of experiments were run: (1) The normal enzyme activity was determined; (2) carbonic anhydrase B was substituted for the normal enzyme: (3) carbonic anhydrase B was added to the normal enzyme. Experiments were run in triplicate with 3—5 different substrate concentrations. Spectrophotometric measurements were performed with a Gilford model 2400 spectrophotometer and spectrofluorometric determinations were achieved with an Aminco-Bowman spectrophotofluorometer.

Since a number of the non-physiological systems involved the addition or withdrawal of water, we began our search with substrates known to be utilized in hydration and dehydration reactions of intermediary metabolism. Among the substrates we tested were L-serine, fumarate, *cis*-aconitate, citrate, phosphogluconolactone, D-gluconolactone, acetyl-CoA, acetoacetyl-CoA, β-glucuronides, D-glyceric acid-2-phosphate, L-glutamine, and fructose 1,6-diphosphate. The reactions studied and the methods used are listed in Table I. Our results were negative for all of the hydrations and dehydrations listed. At concentrations up to 20 μM carbonic anhydrase, no catalytic activity could be demonstrated.

Hughes [15] has reviwed studies suggesting that hydrolytic metalloenzymes can catalyze a variety of decarboxylase reactions by Lewis and acid catalysis; the metal ion facilitates the removal of the CO_2 group by its ability to accept electron pairs. Since carbonic anhydrase is a zinc containing enzyme, and zinc is a good example of a strong Lewis acid catalyst, the possibility existed that

TABLE I

Reaction	Enzymic activity	Ref. for methods
1. L-Serine → Pyruvate + NH_3	Serine dehydratase (EC 4.2.1.13)	27,28
2. Fumarate + H_2O → L-Malate	Fumarase (EC 4.2.1.2)	29
3. Isocitrate → Cis-Aconitate → Citrate	Aconitase (EC 4.2.1.3)	30
4. 6-Phospho-D-glucono-δ-lactone + H_2O → 6-Phospho-D-Gluconate	6-Phosphogluconolactonase (EC 3.1.1.31)	31,32
5. D-Glucono-δ-lactone + H_2O → D-Gluconate	Lactonase (EC 3.1.1.17)	32
6. Acetyl-CoA + H_2O → Acetate + CoA	Acetyl-CoA Hydrolase (EC 3.1.2.1)	33
7. Acetoacetyl-CoA + H_2O → Acetoacetate	Acetoacetyl-CoA Hydrolase (EC 3.1.2.11)	33
8. β-D-Glucuronides + H_2O → D-Glucuronates + Alcohol	β-Glucuronidase (EC 3.2.1.31)	34—36
9. D-Glyceric acid-2-phosphate → Phospho-*enol*pyruvate + H_2O	Enolase (EC 4.2.1.11)	37
10. L-Glutamine + H_2O → L-Glutamate	Glutaminase (EC 3.5.1.2)	38
11. γ-Glutamyl-*p*-nitroanilide + glycylglycine → *p*-nitroanilide + glutamylglyoylglycine	γ-Glutamyltranspeptidase (EC 2.3.2.2)	39
12. L-Glutamine + hydroxylamine → Glutamylhydroxamate + NH_4^+	D-Glutamyltransferase (EC 2.3.2.1)	40
13. *p*-Nitrophenylphosphate + H_2O → p-Nitrophenol phosphoric acid	Alkaline and acid phosphatase (EC 3.1.3.1.) and (EC 3.1.3.2)	41
14. Fructose 1,6-diphosphate → dihydroxyacetone phosphate + D-Glyceryldehyde 3-phosphate	Aldolase (EC 4.1.2.13)	42
15. Histidine → Histamine + CO_2	Histidine decarboxylase (EC 4.1.1.22)	43
16. Ornithine → Putrescine + CO_2	Ornithine decarboxylase (EC 4.1.1.17)	44
17. Pyridoxine → Pyridoxal 5′-phosphate	Pyridoxal kinase (EC 2.7.1.35)	22,23
18. Pyridoxal 5′-phosphate → Pyriodoxal	Pyridoxin dehydrogenase (EC 1.1.1.65)	22,23

the enzyme might possess decarboxylase activity. The choice of histidine decarboxylation to test this possibility, was based on the structural suitability of the imidazole nucleus for binding to the active site. It has been shown that imidazole is a rather specific inhibitor of carbonic anhydrase B [16,17]. Our results were negative with respect to the ability of carbonic anhydrase to facilitate the removal of CO_2 from histidine.

Negative results were also obtained in studies of ornithine decarboxylase activity. We were prompted to try this substrate because of the reports that ornithine decarboxylase activity fluctuates in response to a variety of hormonal stimuli [18]; similar changes in carbonic anhydrase levels have been demonstrated with hormonal alterations. [19,20].

Pocker and Meany [12] have established that bovine carbonic anhydrase catalyzes the hydrations of 2-, 3- and 4-pyridine aldehydes. Although the pyridine containing compounds which they studied were 'artificial' substrates, they are of similar structure to the major forms of vitamin B_6. The dietary usefullness of pyridoxine, pyridoxal and pyridoxamine of plant and animal origin depends on their conversion into the metabolically active coenzyme, pyridoxal phosphate. The details of the conversions of B_6 compounds in human erythrocytes are not fully established [21] but the structural similarity to 'artificial' pyridine substrates and the fact that these reactions take place in the red cell, prompted our study of the effectiveness of carbonic anhydrase in vitamin B_6 metabolism. Pyridoxine metabolism was studied under the conditions of

incubation described by Wada et al. [22] and the formation of pyridoxal and pyridoxal 5'-phosphate was determined fluorimetrically by the method of Srivastava and Beutler [23]. At concentrations of up to 20 μM carbonic anhydrase, no catalytic activity could be demonstrated.

In conclusion, although it appears that enzyme B does not function in vivo as a carbonic anhydrase (i.e. in red cells with HCO_3^- or CO_2 as substrate), our results with 18 other possible physiological substrates were negative.

In recent studies of the phylogeny of red cell carbonic anhydrase, it was suggested that a gene duplication may have occurred some 10^8 years ago giving rise to the two human red cell enzymes now found [24,25]. Before the age of mammals, there appears to have been a progression from a low to high activity form, which may represent an improvement or refinement of the enzyme in response to vertebrate evolution [25]. In this context, the possibility exists that mammalian carbonic anhydrase B is a relatively late evolutionary accident with no physiological tole. This concept is supported by many reports that the concentration of B varies widely in man but C does not [24]; one family of Greek origin has been found to have an inherited absence of red cell carbonic anhydrase B [26]. On the other hand, it seems plausible that the second most abundant protein in red cells might have a function, and the existence of non-CO_2 substrates makes a continuing search of theoretical as well as of practical interest. We intend to study additional substrates, both of purely chemical as well as physiological interest.

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