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Novel inhibition of carbonic anhydrase isozymes I, II and III by carbamoyl phosphate

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Carbamoyl phosphate has been shown to inhibit carbonic anhydrase (CA) isozymes CA I, CA II and CA III. This physiologically important molecule is the most potent, naturally occurring inhibitor of carbonic anhydrase yet found. It is also unique, among carbonic anhydrase inhibitors discovered hitherto, in that it inhibits the 3 isozymes with equal effect, despite their strikingly different properties. The results imply the participation of carbonic anhydrase in the regulation of substrate availability for the urea cycle.

Carbonic anhydrase isozyme *Carbamoyl phosphate* *Carbamoyl phosphate synthetase* *Enzyme inhibition* *Urea cycle*

1. INTRODUCTION

Carbonic anhydrase (EC 4.2.1.1) is a monomeric, zinc metalloenzyme which physiologically catalyzes the reversible hydration of CO_2. In addition it acts as a general acid-base catalyst towards a number of 'synthetic' substrates such as *p*-nitrophenyl acetate [1–3]. It is abundantly present in a wide variety of organisms and is considered to be of fundamental importance in several different processes including calcification, respiration, and transport and accumulation of H^+ and HCO_3^- [4,5].

In mammals, birds and reptiles, 3 different isozyme forms, designated CA I, CA II and CA III, have been identified [6–8]. These exhibit strikingly different properties in terms of structure, particularly at the active site, catalytic activity and inhibition parameters [6–11]. The CA II isozymes have substantially the highest levels of CO_2 hydratase and esterase (*p*-nitrophenyl acetate) activities. In contrast, the CA III isozymes, abundant in red skeletal muscle and also present in liver of some mammals [6,7,9,12], possess notably low activities, whilst additionally possessing a novel low acid phosphatase activity [13], which tempts speculation that they fulfill an as yet unknown physiological role other than the catalysis of CO_2-hydration.

Consideration of the effects of the various carbonic anhydrase inhibitors hitherto examined reveals a similarly varied picture when the isozymes are compared [9,10]. CA II, the major respiratory and secretory enzyme form in animals, is the isozyme most sensitive to inhibition by heterocyclic or aromatic sulphonamides, whilst CA III has a relatively low affinity for such inhibitors. On the other hand, inhibition by halides follows a different pattern, CA II being relatively insensitive, whilst CA I and CA III are more readily inhibited. Exceptionally, the cyanate anion inhibits CA III the most strongly of all 3 isozymes [9,10].

We describe here the effects of carbamoyl phosphate on the 3 isozymes of carbonic anhydrase. Carbamoyl phosphate is the first naturally occurring, physiologically important molecule found to inhibit carbonic anhydrase activity.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Carbamoyl phosphate and *p*-nitrophenyl acetate were obtained from Sigma, MO. All other reagents were analytical grade.

2.2. *Enzyme purification*

Human CA I and CA II were purified from red cell haemolysates by affinity chromatography as in [14]. Human CA III was prepared from post-mortem specimens of psoas major muscle by affinity chromatography followed by gel filtration as in [15].

Bovine and chicken CA III from skeletal muscle were first separated from CA I and CA II isozymes by affinity chromatography [14] followed by DEAE-cellulose chromatography and gel permeation using Sephadex G-75.

Rat liver CA III was purified as in [12].

2.3. *Enzyme activity measurements*

CO_2 hydratase activity was measured using a pH-stat assay system comprising a PHM 64 pH meter, a TTT 60 titrator and a 2.5-ml ABU 80 burette, all from Radiometer, Copenhagen. The assays were performed in a total volume of 10.0 ml, thermostatted at 2°C. The reaction was measured in the direction of bicarbonate dehydration, essentially as in [16,17], at pH 7.1, using 1.0 M sulphuric acid as titrant. The reaction mixture comprised 6.7 mM Na_2HPO_4, 6.7 mM NaH_2PO_4, 30 mM $NaHCO_3$ and 0.5 mM EDTA. The product of the reaction (CO_2) was removed by continuously bubbling nitrogen uniformly through the sintered glass floor of the reaction vessel.

Esterase activity toward *p*-nitrophenyl acetate was measured spectrophotometrically at 348 nm as in [18]. The reaction mixture comprised 30 mM sodium diethylmalonate buffer (pH 7.2) and 1 mM *p*-nitrophenyl acetate in a total volume of 1.0 ml.

3. RESULTS

Carbamoyl phosphate inhibits both the bicarbonate dehydration and the esterase activities of the 3 isozymes of carbonic anhydrase. A concentration of 25 mM achieved total inhibition of human red cell CA I and CA II, CA activity in a total human red cell lysate, and CA III from human, bovine, sheep and chicken skeletal muscle and rat liver.

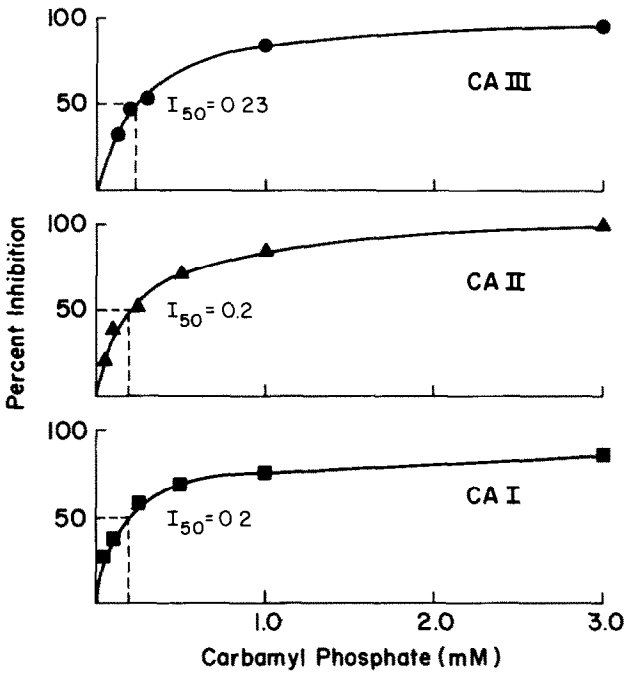

Fig. 1. Effect of carbamoyl phosphate on the inhibition of carbonic anhydrase isozymes CA I and CA II from human red cell and CA III from rat liver. Enzyme activity was measured as dehydration of bicarbonate at pH 7.1, using a pH-stat assay system as described in the text. Enzyme concentrations in each set of assays were: CA I, 90 μg/ml; CA II, 0.96 μg/ml; CA III, 0.38 mg/ml.

Fig. 1 shows the effect of carbamoyl phosphate concentration on the inhibition of the bicarbonate dehydration activity of human red cell CA I and CA II and rat liver CA III. Clearly, the I_{50} of ≃ 0.2 mM is the same for each isozyme.

The effects of carbamoyl phosphate concentration on the esterase activity toward *p*-nitrophenyl acetate of human and sheep CA III were also examined and similar results and I_{50} values were obtained.

These results show that carbamoyl phosphate is the most potent naturally occurring inhibitor of carbonic anhydrase yet discovered. It is considerably more inhibitory than Cl^- [19,20] and its potency of CA III inhibition is comparable to that of acetazolamide [9,21].

4. DISCUSSION

The inhibition of carbonic anhydrase by carbamoyl phosphate is of significant interest both to the molecular enzymologist and in the broader metabolic arena. The most striking feature, in enzymological terms, is the uniform degree of inhibition among the different isozyme forms. All previously published work on carbonic anhydrase inhibitors shows a considerable variation in degree of inhibition from one isozyme to another [4,10]. In the case of the sulphonamides, the most powerful known inhibitors of carbonic anhydrase, the K_i values for the different isozymes range by a factor approaching 10^4, with CA III showing a remarkable resistance to sulphonamide inhibition in comparison with the other two isozymes [4,9,10,21]. CA II is about 10-times more sensitive than CA I to acetazolamide and sulphanilamide, but shows less difference in response to other sulphonamides [4,10]. Conversely, when we consider the effect of halide inhibitors, we find that CA is is about 100-times more sensitive to inhibition than CA II, and is similar to CA III in this respect [9,10].

Considered against this background, the identical inhibition of all 3 isozymes by carbamoyl phosphate is remarkable. This is especially so with regard to CA III, which shows fundamental differences in active site composition by comparison with the other two isozymes [11]. Of the 5 putative active site residues unique to CA III, two (positions 67, 91) are arginines, which might well be expected to interact with phosphorylated modulators [22]. Thus, the equivalent inhibition of the 3 isozymes suggests that carbamoyl phosphate may be binding elsewhere than at this isozyme-specific region of the active site.

The uniform nature of inhibition also provides confirmatory evidence that carbamoyl phosphate is a genuine inhibitor. At alkaline pH, carbamoyl phosphate breaks down to give cyanate and phosphate [23–25]. The possibility of the inhibition observed here being due to cyanate contamination is however excluded, since this anion shows different degrees of inhibition with each isozyme [10,19].

The metabolic implications of carbamoyl phosphate inhibition are intriguing. Despite the difficulties of measuring tissue levels of carbamoyl phosphate, due to the instability of the molecule during extraction [26,27], intramitochondrial levels as high as 22 $nmol \cdot mg^{-1}$ mitochondrial protein, a level clearly inhibitory to carbonic anhydrase, have been recorded using a rapid, fluorometric method [27]. Similarly inhibitory levels have been recorded in the mitochondrial matrix [28].

The presence of carbonic anhydrase in the matrix of liver mitochondria has been demonstrated [29,30] and a role for this enzyme in urea synthesis has been suggested [31]. Inhibition of liver mitochondrial carbonic anhydrase by acetazolamide reduces citrulline synthesis by more than 70% despite the fact that the uncatalyzed rate of CO_2 hydration is greatly in excess of the rate of HCO_3^- consumption by carbamoyl phosphate synthetase I (CPS I) at maximum flux. Consequently it has been suggested that access of HCO_3^- to CPS I may be regulated by carbonic anhydrase and the two enzymes could possibly be complexed together [31]. If they do indeed function in some form of control unit, it is interesting to note that carbamoyl phosphate, whilst inhibiting both enzymes, is clearly a more potent inhibitor of carbonic anhydrase than of CPS I, for which the K_i is 10–19 mM [32].

The possibility that carbonic anhydrase is involved in control of the activity of carbamoyl phosphate synthetase II (CPS II), the cytoplasmic enzyme which functions exclusively in pyrimidine biosynthesis, seems remote in view of the low cytoplasmic levels of carbamoyl phosphate, the low activity of CPS II and the tight structural and allosteric control under which it is held [33–35]. However, the likely importance of carbonic anhydrase in the regulation of substrate availability for the urea cycle clearly merits deeper investigation.

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