INACTIVATION OF HUMAN ERYTHROCYTE CARBONIC ANHYDRASES BY BROMOPYRUVATE

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1. Introduction

The two main forms of the human erythrocyte carbonic anhydrase (EC 4.2.1.1), generally denoted B and C, are both metalloenzymes requiring zinc for activity and have a molecular weight around 30,000 [1,2]. They catalyze the reversible hydration of carbon dioxide and carbonyl groups of some aldehydes and ketones [3] as well as the hydrolysis of certain esters. The enzymes are powerfully inhibited by aromatic and heterocyclic sulfonamides and, more weakly, by monovalent anions. Enzyme B differs from the C form in catalytic properties implying structural dissimilarities in the active sites. In enzyme B two histidines of the active site region have been identified by chemical modification; one is modifiable with bromo- or iodoacetate, and 1-fluoro-2, 4-dinitrobenzene [4–7], the other with *N*-chloroacetylchlorothiazide* [8], a sulfonamide carrying a reactive group. Human carbonic anhydrase C is the only form of the enzyme characterized by high-resolution X-ray diffraction [9] but has been less extensively studied by chemical modification. A histidine side chain in the active-site region has been labelled with bromoacetazolamide [10].

The present paper describes the action of bromopyruvate on human carbonic anhydrases B and C. Both enzymes can be chemically modified apparently in a specific reaction taking place with a histidine residue at or near the active site. The covalently modified enzymes seem to possess residual enzymic activity, and may be useful for studies of enzymic mechanism as demonstrated for the C enzyme derivative in a recent kinetic characterization [11].

* *Abbreviations:*
Salamide: 5-chloro-2, 4-disulfamoyl-anilin,
Ethoxzolamide: 6-ethoxy-2-sulfamoyl-1, 3-benzothiazole,
N-Chloroacetylchlorothiazide: 6-chloro-7-*N*-chloroacetylsulfamoyl-1,2,4-benzothiadiazine 1,1-dioxide.

2. Materials and methods

Human carbonic anhydrases B and C were prepared from erythrocytes [12] avoiding lyophilization for enzyme (cf. [13]).

Enzyme activity was determined from the rate of hydrolysis of *p*-nitrophenyl acetate at 25°; for enzyme B in Tris-sulfate, pH 7.6, I = 0.075; for enzyme C in 0.05 M phosphate buffer, pH 6.8. 25 µl of 40 mM ester in acetone was added to the assay solution (2.50 ml) to start the reaction. The change in absorbance was followed at 400 or 348 nm [14]. The enzyme concentration was generally 1–4 µM. Under these conditions the concentration of ester substrate is far below the K_m-value [14] and enzyme inhibition was treated as non-competitive with respect to substrate. Amino acids were analyzed after hydrolysis of the protein or peptide in 6 M HCl for 20 hr at 110° [15]. 3′-Carboxyketoethylhistidine was determined after conversion to 3′-carboxymethylhistidine [16]. Modified histidine was located in the amino acid sequences of the enzyme using similar methods as described elsewhere [17].

Bromopyruvic acid from K and K Laboratories Inc., Hollywood, Calif., USA was used without further purification.

Table 1
Reversible inhibition of human carbonic anhydrases B and C with bromopyruvic acid and related compounds.

	Enzyme B (pH 7.6)			Enzyme C (pH 6.8)		
Inhibitor	Concentration of inhibitor (mM)	Esterase activity (%)	K_I (mM)	Concentration of inhibitor (mM)	Esterase activity (%)	K_I (mM)
Bromopyruvic acid	0	100	–	0	100	–
	0.66	82.4	3.1	8.9	73.0	24
	1.3	70.0	3.1	17.8	58.0	24
	2.6	57.8	3.5	26.7	47.2	24
	6.6	28.3	2.6	35.6	39.2	23
		Mean value	3.1		Mean value	24
Pyruvic acid			5.7			44
α-Bromopropionic acid			3.6			38
Propionic acid			11			48

Dissociation constants (K_I) for enzyme–inhibitor complexes were estimated from enzyme activity measurements on the rate of hydrolysis of p-nitrophenylacetate as described in the Materials and methods section.

3. Results

The data in table 1 show that bromopyruvate can act as a reversible inhibitor of carbonic anhydrases B and C. For both enzymes the reversible complex with bromopyruvate seems to be completely inactive as judged from the invariant value of the dissociation constant (K_I) for the complex obtained at different concentrations of bromopyruvate (cf. [4]). The comparisons of bromopyruvate with the other compounds in table 1 show that the presence of a bromine atom in the molecule increases the binding strength and a similar effect is exhibited by the carbonyl group.

Prolonged incubation with bromopyruvate leads to a decrease of enzymic activity which cannot be restored by dilution or dialysis. This irreversible inactivation of enzymes B and C is illustrated in figs. 1 and 2 with the experimental conditions specified in the captions to figures. Enzyme B reacted for 1 hr has a residual activity of about 4% and contains one equivalent of 3'-derivative of histidine. The histidine modified was found to be identical to the residue modifiable with bromo- and iodoacetate, and dinitrofluorobenzene, which has tentatively been assigned to position 204 in the enzyme sequence (see [1]).

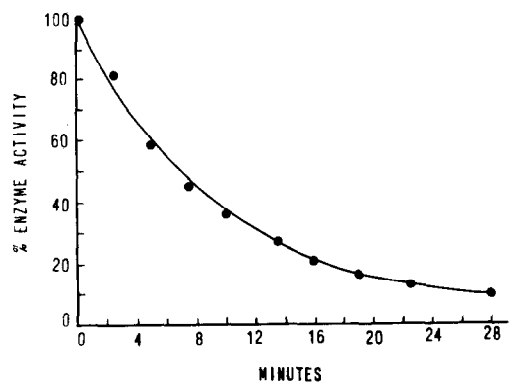

Fig. 1. Inactivation of human carbonic anhydrase B with bromopyruvate. Reaction conditions: 25°; Tris-sulfate buffer, pH 7.6, $I = 0.075$; 0.15 mM carbonic anhydrase; 3.0 mM bromopyruvate. From the K_I for the reversible inhibition with bromopyruvate (see table 1) 50% of the enzyme should be present as reversible complex with inhibitor at the start of the reaction. Aliquots of the reaction mixture were withdrawn at various times and assayed for enzyme activity as described in the Materials and methods section. Half-reaction time for the loss of activity is 7 min.

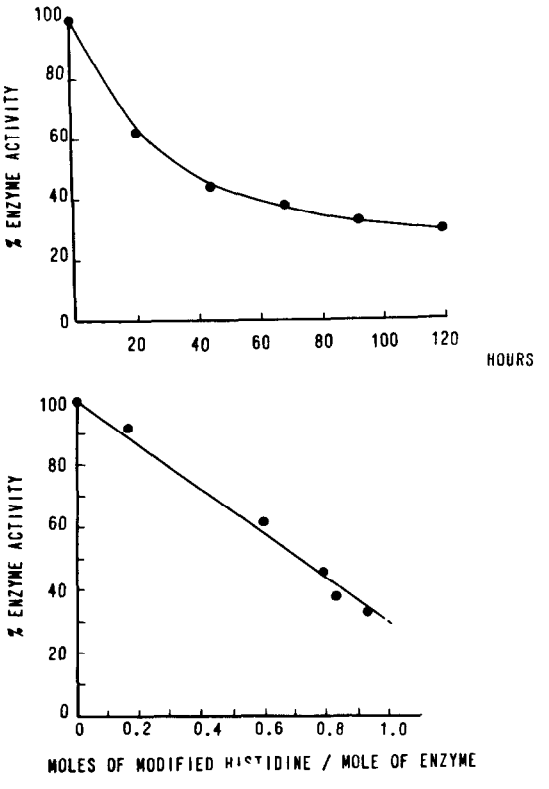

Fig. 2. Inactivation of human carbonic anhydrase C with bromopyruvate. Reaction conditions: 25°; 0.05 M phosphate buffer, pH 6.8; 0.12 mM carbonic anhydrase; 25 mM bromopyruvate. From the K_I for the reversible inhibition with bromopyruvate (see table 1) about 50% of the enzyme should be present as reversible complex with inhibitor at the start of the reaction. Aliquots of the reaction mixture were withdrawn at various times and used for determination of enzyme activity and amino acid composition of reacted protein as described in the Materials and methods section.

For enzyme C (fig. 2), the decrease in enzymic activity is slow under the conditions used and levels off at about 30% of residual activity. It correlates with the formation of one equivalent of modified histidine (see fig. 2), suggesting that a specific residue becomes modified. The following peptide preparation was isolated from a tryptic digest of modified enzyme: Lys Asx_{6-7} Ser Glx_3 Gly_2 Ala His (modified) Val Ile Leu Phe_2. It can be fitted into the sequence of protein covering the stretch from residue 58 to 76 (see [1]). This portion of the sequence has not yet been worked out in detail but comparisons with other forms of carbonic anhydrase [18] suggest that the histidine residue would occupy position 64 in the enzyme sequence *.

The rate of formation of inactivated enzyme C was studied at various concentrations of bromopyruvate (see table 2). Apparent first-order rate constants (k) were estimated for the loss of activity and found to be proportional to the fraction of enzyme present as reversible complex with reagent (R) in consistency with the following scheme:

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k'} \text{Inactivated enzyme}$$

where K_1 is the dissociation constant for the reversible binding of bromopyruvate and k' the first-order rate constant for the conversion of the reversible complex into chemically modified enzyme.

Fig. 3 shows the influence of various enzyme inhibitors on the inactivation of carbonic anhydrase C with bromopyruvate. The K_I values for salamide and $KAu(CN)_2$ determined here were 1 and 1000 μM, respectively. For ethoxzolamide, the K_I value was found to be too low to be possible to estimate with the assay used here ($K_I \ll 1$ μM) (cf. [2]). Fig. 3 shows that the sulfonamide inhibitors salamide and ethoxzolamide partially protect the enzyme while the anionic complex $Au(CN)_2^-$ accelerates the inactivation.

4. Discussion

Human carbonic anhydrase B can be specifically modified with bromo- and iodoacetate [4–6], iodoacetamide [4, 6] and, as shown recently in our laboratory, DL-α-bromocaproate [19]. These reagents bind as reversible inhibitors to the active site and a particular histidine residue, supposedly near the position for reversible binding, becomes modified. Bromopyruvate shows in its action on human enzyme B cer-

* The numbering of residues in human enzyme C used here differs from that of Liljas [9] who uses number 63 for this histidine.

Table 2
Inactivation of human carbonic anhydrase C at different concentrations of bromopyruvate.

Concentration of reagent (mM)	R	$k(hr^{-1} \times 10^3)$	$k'(hr^{-1} \times 10^3)$
8	0.25	13.7	55
17	0.41	24.2	61
35	0.58	28.7	50
53	0.68	39.6	58
71	0.74	43.0	58
		Mean value	56

R is the fraction of enzyme with bromopyruvate reversibly bound at the start of the experiment as estimated from the K_I value in table 1. First- order rate constants (k) for the formation of inactivated enzyme were estimated at various concentrations of reagent. k' has been calculated as k/R and should correspond to the rate constant for the conversion of the enzyme–inhibitor complex to chemically modified enzyme as in the scheme shown in the text. The value of $k' = 0.056\ hr^{-1}$ found here corresponds to a helf-reaction time for this conversion of 12.3 hr. The experiments were performed at 25° in 0.05 M phosphate buffer, pH 6.8 with an enzyme concentration of 0.10 mM. Enzyme activity was measured under the same conditions using diluted aliquots (see Materials and methods section).

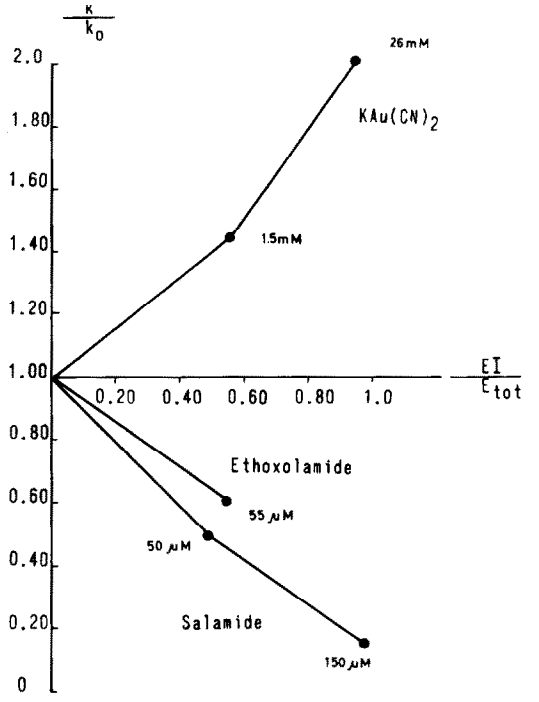

Fig. 3. Effects of various carbonic anhydrase inhibitors on the inactivation of human carbonic anhydrase C with bromopyruvate. Reaction conditions: 25°; 0.05 M phosphate buffer, pH 6.8; 0.10 mM carbonic anhydrase; 25 mM bromopyruvate. Samples were taken after various times and assayed for enzymic activity after dilution (see Materials and methods section). k and k_0 are the first-order rate constants estimated for the loss of enzymic activity in the presence and absence of inhibitor, respectively. EI/E_{tot} represents the fraction of enzyme occurring as reversible complex with added inhibitor assuming noncompetitive binding with respect to bromopyruvate. For 1.5 mM $KAu(CN)_2$, 50 and 150 μM salamide, samples of the reaction product after 69 hr of reaction time were also analyzed for modified histidine. The results obtained fitted well with the relation between modified histidine and enzymic activity shown in fig. 2.

tain similarities with these reagents. The same histidine residue becomes modified and the modified enzyme appears to have a certain amount of residual activity although the reversible enzyme–inhibitor complex behaves as completely inactive. These similarities would suggest common mechanistic features and the discussions of the action of bromoacetate, iodoacetate, and iodoacetamide on human enzyme B [4–6] might to a considerable extent also apply to bromopyruvate. With bromopyruvate the inactivation reaction is significantly faster than with the other reagents in accordance with the expectation that the halide atom in bromopyruvate should be a better "leaving group".

Table 2 shows that the rate of inactivation of enzyme C is not proportional to the concentration of reagent but to the fraction of enzyme present as reversible complex with bromopyruvate. This finding suggests that the modification takes place in the active-site region which is further supported by the data in fig. 3 illustrating that enzyme inhibitors influence the rate of inactivation. The high residual activity of the reacted enzyme shows that the modifiable histidine does not partake in any fundamental fashion in the catalytic reaction. Since the reversible complex, as judged from the data in table 1, appears to be completely inactive the high residual activity would also suggest that the covalently attached carboxyketoethyl group does not occupy a position identical to that held by the reagent in the reversible com-

plex. It is also notable that the formation of irreversibly modified enzyme is a slow reaction (cf. the rate of inactivation of enzyme B).

The present knowlegde of the primary structure of the human carbonic anhydrases indicates that histidine 67 (modifiable with *N*-chloroacetylthiazide) and histidine 204 (modifiable with bromo- and iodoacetate, iodoacetamide, dinitrofluorobenzene, and bromopyruvate) in the B enzyme are replaced by other residues in enzyme C [1,17]. Both enzymes appear to have a histidine residue at position 64 but only in enzyme C has this residue been modified with bromopyruvate. Assuming the same rate of reaction in both enzymes, the modification of histidine 64 in enzyme B might have escaped detection. It is also possible that the carboxyketoethyl group introduced in the active site of enzyme B through the rapid reaction with histidine 204 prevents the modification of histidine 64. The results from chemical modifications are thus possible to explain without invoking conformational differences between enzyme B and C. Combined with X-ray data for crystalline enzyme C [9,1] they even provide evidence for similarities between the enzymes in peptide chain stretching in the region of the active site. According to the X-ray model, residues 64, 67, and 204 should namely all be parts of the active site region.

The X-ray results suggest that histidine 64 in native enzyme C is hydrogen-bonded to an ordered arrangement of water molecules present in the active-site cavity [9,1]. This arrangement also involves the metal ion at the bottom of the cavity which has been ascribed a central role for the catalytic reaction. Inhibitor molecules have in the X-ray studies been observed to bind at or close to the metal ion causing a disruption and a partial or complete displacement of the ordered water. Anionic inhibitors have been reported to bring about a change in the properties of histidine 64 observed as an increased tendency of this residue to bind methylmercurithioglycolic acid [9]. This change, possibly due to a release of the hydrogen bonding or resulting from a small conformational shift, may cause the increase in reactivity towards bromopyruvate noted here in the presence of $Au(CN)_2^-$ (fig. 3).

Sulfonamide inhibitors bind to carbonic anhydrase with the sulfonamide group oriented towards the metal ion [1]. Salamide has 2 sulfonamide groups and X-ray studies on its complex with enzyme C suggest that the second one (in *meta*-position) is hydrogen-bonded to histidine 64 [1,9]. This may explain the protective effect of salamide against inactivation with bromopyruvate (see fig. 3). It is also possible that filling up the active-site cavity with a bulky inhibitor could result in a stereochemical hindrance and this might be the mechanism behind the protective effect found for ethoxzolamide. The considerably smaller sulfonamide inhibitor, acetazolamide, does not show significant protection, if any [19], consistent with the X-ray evidence suggesting that in this enzyme–inhibitor complex histidine 64 remains accessible [9].

Several questions, many of these requiring further experiments to be answered, can be raised about the reversible binding of bromopyruvate. As a monovalent anion bromopyruvate would be expected to show affinity for the ordinary inhibitor site at or close to the metal ion. The absence of protective effect found for $KAu(CN)_2$ and acetazolamide would, however, suggest that a reversible binding of a bromopyruvate molecule at the ordinary inhibitor site is not a prerequisite for it to modify. Instead, it appears that the inhibitor position, defined as for $KAu(CN)_2$ and acetazolamide, does not overlap the space required for a bromopyruvate molecule to react with histidine 64. Bromopyruvate differs by containing a carbonyl group from the inhibitors so far studied by X-ray diffraction. The active site of carbonic anhydrase is known to interact with carbonyl groups, namely in the catalytic reaction. At present, the possibility cannot be excluded that bromopyruvate in the active site becomes oriented as a substrate instead of, or in addition to being reversibly bound as an anionic inhibitor. The finding that the enzyme can act as a catalyst in a closely related system, the dehydration of 2, 2-dihydroxypropionate to pyruvate, would seem to support such an idea [3].

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