REACTION OF BROMOACETAZOLAMIDE WITH CRYSTALS OF HUMAN CARBONIC ANHYDRASE C

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SUMMARY: Crystals of human carbonic anhydrase C were reacted with [^{14}C] bromoacetazolamide and an alkylation was found to occur at the 3'-nitrogen of histidine-64, which is known to be located in the active site region. This reaction requires that the bromomethyl group of the reagent move from its initial binding position, phenylalanine-129, within bonding distance of histidine-64 without significantly disturbing the interaction of the sulfonamide moiety with the active site zinc ion. Since the same histidine has been found previously to react with bromoacetazolamide when the enzyme was in solution, it can be concluded that the conformation of the active site histidine is the same in the two states.

Bromoacetazolamide is a simple electrophylic derivative of acetazolamide, the latter being a powerful reversible inhibitor of erythrocyte carbonic anhydrases (1), and covalently interacts with histidine-64 (histidine-63 in the recently reported sequence (2)) of human carbonic anhydrase C (3). High resolution x-ray diffraction studies have demonstrated that this amino acid is located in the active site region of the enzyme and approximately 6 A° from the essential zinc ion (4). Prior to the identification of histidine-64 as the sole site of bromoacetazolamide alkylation (3), it had been suggested that the target for this reaction was histidine-129 (5). This suggestion was based on the electrondensity map of the enzyme-acetazolamide complex which indicated that the methyl group of the inhibitor is in close proximity to histidine-129 (5). Model building studies seemed to confirm this prediction (6). The need for clarificatic of this significant contradiction prompted us to study the covalent interaction of bromoacetazolamide with human carbonic anhydrase C crystals under conditions similar to those employed in the crystallographic studies.

Methods

Various procedures used in this study have been reported previously: preparation of [^{14}C] bromoacetazolamide (7), purification of the modified enzyme using DEAE cellulose column chromatography (3), isolation of the labelled tryptic peptide from modified enzyme prepared with [^{14}C] bromoacetazolamide (3), amino acid analysis (8), estimation of enzymic activity with respect to the hydrolysis

of p-nitrophenylacetate (8), starch gel electrophoresis (8) and radiocarbon counting (8).

Preparation of Crystals: The isolation of human carbonic anhydrase C differed from reported procedures (8) in that hemoglobin was separated from the enzyme using DEAE Sephadex A-50 as described by Armstrong et al. (9) in the batchwise manner suggested by Liljas (5). Enzyme solutions obtained in this manner were concentrated by ultrafiltration (Amicon, UM-10) rather than by freeze-drying. Crystals were grown according to the procedure of Tilander et al. (10) in which 1% enzyme in 0.05 M Tris chloride buffer is dialysed against the same buffer with a gradually increasing concentration of $(NH_4)_2SO_4$ at 5° and pH 8.5. When an $(NH_4)_2SO_4$ concentration of 2 M was attained, the solution was centrifuged to remove noncrystalline precipitate. Crystallization occurred at an $(NH_4)_2SO_4$ concentration of 2.3 M and the pH of the final suspension was 8.1.

Reaction of Crystalline Enzyme with [^{14}C] bromoacetazolamide: Twenty mg of wet, centrifuged crystals, representing 5 mg of enzyme as determined spectrophotometrically in solution (11), were washed three times with 0.05 M Tris chloride buffer containing 2.3 M $(NH_4)_2SO_4$ at pH 8.1. The supernatant in each case was removed by centrifugation and analyzed for the presence of dissolved enzyme by measuring the optical density at 280 nm. After the second and third washings the amount of dissolved enzyme was found to be less than 0.3%. The crystals were than exposed to a 1.2 fold molar excess of [^{14}C] bromoacetazolamide (0.0602 mg) for 24 hours at 22° and pH 8.1 in 2.5 ml of 0.05 M Tris chloride buffer containing 2.3 M $(NH_4)_2SO_4$. The crystals were subsequently centrifuged to remove an amount of unreacted and unbound reagent equivalent to 10.7%. Resuspension of the crystals in 2.5 ml of the above buffer, from which the [^{14}C] bromoacetazolamide had been omitted and replaced by a 35 fold molar excess (1.375 mg) of acetazolamide, was intended to displace from the enzyme any bound but unreacted reagent. After 24 hours the mixture was again centrifuged and the decanted supernatant contained 33.7% of the total amount of radioactivity initially added. Using additional Tris-$(NH_4)_2SO_4$-acetazolamide buffer, the crystals were then transferred quantitatively to a dialysis tube and dialysed initially for three days against 0.05 M Tris chloride buffer, pH 8.1, containing 0.0025 M acetazolamide plus 2.3 M $(NH_4)_2SO_4$ and subsequently for an additional three days against 1 M NaCl. This latter step, resulting in the dissolution of the crystals, was necessary to remove tightly bound acetazolamide which would otherwise have affected the separation by affinity chromatography of modified from unreacted enzyme. Dialysis was continued for a final 3 days against distilled water to remove NaCl and the solution was subsequently freeze-dried. All dialyses were carried out at 4°.

TABLE I

Covalent Interaction of Human Carbonic Anhydrase C with Bromoacetazolamide under Various Conditions

Experiment	3-CM Histidine Formed	Incorporation of [^{14}C] bromoacetazolamide
	residue/molecule	mole/mole enzyme
Crystalline enzyme + [^{14}C] bromoacetazolamide. After 24 hrs. acetazolamide added.	0.42	0.41
Crystalline enzyme + [^{14}C] bromoacetazolamide *	0.47	0.61
Crystalline enzyme + acetazolamide. After 24 hrs. [^{14}C] bromoacetazolamide added.	0.06	0.08
Dissolved enzyme + [^{14}C] bromoacetazolamide. After 24 hrs. acetazolamide added.	0.46	0.44
Dissolved enzyme + [^{14}C] bromoacetazolamide *	0.45	0.602
Dissolved enzyme + [^{14}C] bromoacetazolamide + $(NH_4)_2SO_4$ *	0.43	0.55

* Reaction time 24 hours

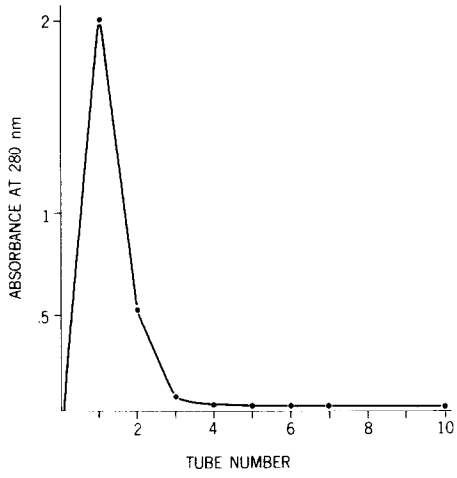

Fig. 1. Purification of human carbonic anhydrase C alkylated with bromoacetazolamide. Alkylated enzyme purified on a DEAE cellulose column was chromatographed on a column (0.9 x 4 cm) of affinity resin (see "Methods") which was previously equilibrated with 0.005 M Tris sulfate buffer at pH 8.1 containing 0.022 M Na_2SO_4. Elution was performed at 23° with the same buffer and Na_2SO_4. The flow rate was 0.5 ml per minute and 7.5 ml fractions were collected.

Affinity Chromatography: Minor amounts of native enzyme still present in Fraction II from the DEAE cellulose column (ref. 3) were removed on an affinity column prepared by coupling p-aminomethylbenzene sulfonamide (Aldrich) to Sepharose 4B according to the procedure of Whitney (12).

Results

Previous observations in these laboratories have shown that carbonic anhydrase binds acetazolamide pseudo-irreversibly (8). During the usual conditions of chromatography as well as dialysis against distilled water the enzyme-inhibitor complex has been found to be relatively stable. In order to prevent further reaction of bromoacetazolamide with the enzyme after the latter has been finally dissolved, a large amount of acetazolamide in 2.3 M $(NH_4)_2SO_4$ was added to the crystals at the end of the reaction to replace the tightly bound reagent. Furthermore, the reaction mixture was dialysed against acetazolamide in the presence of $(NH_4)_2SO_4$ before the crystals were dissolved by dialysis against NaCl. Under the conditions described, human carbonic anhydrase C reacts slowly with bromoacetazolamide. Following acid hydrolysis of the modified enzyme, 3-CM histidine has been found on the analytic ion exchange column to be the sole amino acid derivative (Table I). This observation, as well as the stoichiometric amount of radioactivity present in the modified enzyme (Table I) demonstrates that bromoacetazolamide reacts exclusively with the 3'-nitrogen of a histidine (or histidines) in the crystalline enzyme. The data presented in Table I also

indicate that if the addition of acetazolamide and the subsequent dialysis against acetazolamide is omitted, both the yield of 3-CM histidine and the incorporation of radioactivity are increased. In this case the non-stoichiometric amount of radioactivity present in the modified enzyme indicates the presence of pseudo-irreversibly bound reagent. A control experiment in which acetazolamide was added to the crystals prior to the reagent resulted in no significant reaction (Table I), thereby indicating that the above procedure indeed prevents the alkylation when the enzyme is in solution. This experiment also demonstrates that bromoacetazolamide reacts exclusively in the active site region. In Table I are presented data obtained from the reaction of dissolved enzyme (0.5 mg in 1 ml of 0.05 M Tris chloride buffer) with 1.2 fold molar excess bromoacetazolamide at pH 8.1 in the presence and the absence of $(NH_4)_2SO_4$. The almost equal yields of 3-CM histidine indicate that $(NH_4)_2SO_4$ does not affect the covalent interaction. Although no direct comparison can be made between the rates at which bromoacetazolamide reacts with the crystalline and the dissolved enzymes, it is noteworthy that the amount of 3-CM histidine formed is independent of the physical state of the enzyme (Table I). It would seem that covalent bond formation and not diffusion of the reagent to the active site is the rate limiting factor.

The modified enzyme was separated from unreacted enzyme by DEAE cellulose chromatography (2) and at pH 7.6 possesses 2.4% esterase activity relative to that of the native enzyme. After further purification by affinity chromatography (Figure 1) this value decreased to less than 0.2% indicating that the modified enzyme is essentially inactive. On starch gel this species moves as a single band toward the anode and ahead of the native human carbonic anhydrase C. Following acid hydrolysis, amino acid analysis indicated the presence in the alkylated species of 0.96 eq. of 3-CM histidine and the simultaneous loss of 1.1 eq. of histidine. This is in good accord with the observed incorporation of 0.89 eq. of radioactivity. A tryptic peptide containing the radioactive label was isolated from the modified enzyme in 41.2% yield and exhibited the following amino acid composition: Asp_6 Ser_1 Glu_2 Gly_2 Ala_1 Val_1 $Ileu_1$ Ieu_1 Phe_2 3-CM His_1 Lys_1. This composition corresponds to the recently reported sequence of the enzyme between residues 58 and 76 (3) and to the tryptic peptide isolated from the same enzyme alkylated in solution with the same reagent (2).

Discussion

While this paper was in preparation Henderson *et al.* (2) reported the complete amino acid sequence of human carbonic anhydrase C. According to their description, the 129th amino acid residue is not histidine as tentatively pro-

posed by the x-ray crystallographers (5) but rather phenylalanine.[1] This observation is inconsistent with the suggested manner of acetazolamide binding (4). Unlike the imidazole ring of histidine, the benzene ring of phenylalanine cannot accept or donate the proton required for the formation of a hydrogen bond with the inhibitor. On the other hand, the possibility that a hydrophobic or charge transfer interaction results in a weak binding to phenylalanine-129 cannot be ruled out at the present time. Indeed, the methyl group of acetazolamide could lie in the vicinity of phenylalanine-129 without participating in any chemical interaction. Such weak binding or lack of it notwithstanding, the reported position of the methyl group of acetazolamide would appear to be closer to phenylalanine-129 than to histidine-64. It would seem, therefore, that the bromomethyl group of bromoacetazolamide would be required to shift somewhat in order to be within covalent bonding distance of histidine-64. Although small movements of the inhibitor cannot be ruled out (13) it seems unlikely that any such movement significantly changes the relative positions of the sulfonamide moiety of the reagent and the essential zinc ion of the enzyme. This follows from the observation that the visible absorption spectrum of the Co^{++}-bovine enzyme alkylated at histidine-64 with bromoacetazolamide is essentially identical to that of the reversible complex with acetazolamide (3). Alternatively, it is possible that the acetylamino moiety of acetazolamide and consequently the bromoacetylamino moiety of bromoacetazolamide lie much closer to histidine-64 in the respective complexes than the present 3 A° electrondensity map of the enzyme-inhibitor complex suggests. X-ray crystallographic studies at a resolution higher than 3 A°, now in progress in Uppsala (13), will hopefully clarify this interesting point.

The observation that bromoacetazolamide reacts with the histidine residue of human enzyme C in both the solution and crystalline forms is evidence that the conformation of the active site around histidine-64 is similar in the two states.

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[1] In the following discussion it is assumed that this amino acid residue and not tyrosine-126 as proposed by Henderson et al. (2) corresponds to histidine-128 in the x-ray model (6,13).

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