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IRREVERSIBLE INHIBITION OF CARBONIC ANHYDRASE BY THE CARBON DIOXIDE ANALOG CYANOGEN

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SUMMARY: Cyanogen (C_2N_2), a molecule with properties remarkably similar to carbon dioxide, differentially inhibits three of the four carbonic anhydrases reported here. Bovine carbonic anhydrase II shows 97% loss of esterase activity with no concommitant loss in hydratase activity. The hydratase and esterase activities of human carbonic anhydrase I are decreased by 80% and 55% respectively. Canine carbonic anhydrase shows similar results to human carbonic anhydrase I, retaining 29% hydratase and 62% esterase activity. Rabbit carbonic anhydrase sustained no loss of either hydratase or esterase activity. This inhibition occurs by an irreversible modification of the enzymes. The kinetic parameters for modified and unmodified enzymes were altered in a way that reflects the characteristic effect for each carbonic anhydrase.

We report here the irreversible inhibition of carbonic anhydrase (4.2.1.1) by the widely distributed environmental contaminant, cyanogen. The process appears to be an active-site-directed chemical modification. Cyanogen (ethanedinitrile, C_2N_2) is widely distributed in the environment from incomplete combustion of nitrogenous compounds. It is a constituent of tobacco smoke, from combustion of non-nitrogenous compounds under conditions where NO_x are formed and from chemical processes. In spite of its well-known high reactivity with several classes of organic compounds (1), there appears to have been no systematic study of its reactions with biologically significant substances. However, it has been proposed that C_2N_2 was a primordial condensing agent on the pre-biotic earth (2); it was shown that C_2N_2 could effect the condensation of phosphate and ribose (3), phosphate and glucose (4), phosphate and nucleoside (5), and between purine precursors (6). In spite of its high reactivity towards various compounds, it hydrolyzes slowly in water. Its aqueous solution show breakdown over a period of many days (1). The chemistry of cyanogen has been reviewed extensively in an older review (1) and more briefly by Ciganek et al. (7).

Many active site directed analogs for affinity labeling have been reported for carbonic anhydrase which generally modify $N\pi$ or $N\tau$ of HIS residues of the active site (8);

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some of them are good structural analogs of HCO_3^-. No reasonable structural analog of CO_2 has been reported to date which inhibits the enzyme and is also an affinity label; the linear N_2O molecule has overall dimensions similar to CO_2. It has been reported to bind and inhibit (9); another study failed to confirm this report (10).

The widely distributed carbonic anhydrase catalyzes

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

We report here the irreversible modification of four mammalian erythrocyte enzymes by low concentrations of C_2N_2 (for a recent review, see Ref. 11).

Materials and Methods

Enzymes and Reagents. Bovine carbonic anhydrase was prepared from bovine blood by the method of Osborne and Tashian (12). It was then purified by affinity electrophoresis as defined by Bergenhem (13). The isolated bovine carbonic anhydrase was found to be pure by SDS gel electrophoresis and isoelectric focussing. The preparation was 100% active by the measurement of acetazolamide inhibition by the method of Pocker (14). The erythrocyte rabbit, dog and human B carbonic anhydrases were bought from Sigma and used without further purification. Acetazolamide, p-aminomethylbenzenesulfonamide, p-nitrophenyl acetate, and p-nitrophenol were obtained from Aldrich.

Esterase Assays. These were carried out by the method of Pocker (14). HEPES buffer was used in the place of Tris- chloride pH 7.2 to avoid inhibition by chloride ions (15).

Hydratase Assays. These were done according to the procedure of Khalifah (10). MOPS buffer pH-7.2 was used in the place of imidazole buffers to avoid the inhibitory action of imidazole. p-Nitrophenol was used as the indicator. Carbon dioxide solutions were prepared by allowing CO_2 to bubble into water for over two hours. The saturated CO_2 solution was then diluted with pH 7 water.

Cyanogen Treatment. C_2N_2 was obtained from Matheson Scientific and showed no measurable HCN when examined by mass spectrometry. The cyanogen was bubbled into pH 4 water until saturation. The molarity at saturation is .14M. This concentration was verified by hydrolyzing an aliquot in aqueous sodium hydroxide and measuring cyanide with calibrated cyanide electrodes. The cyanogen at a 100:1 molar ratio to the carbonic anhydrases was added to the enzymes in pH 4 aqueous solutions and allowed to incubate for two hours at room T($\sim 21^{\circ}$); concentrations of carbonic anhydrase in each case is shown in Tables I and II. Total volumes were less than 1 ml. The cyanogen was removed by entrainment by gently passing nitrogen over the top of the solution. The uninhibited carbonic anhydrases were treated in the same fashion.

Aliquots of headspace of cyanogen solutions were analyzed by mass spectrometry for C_2N_2 and HCN both of which have high vapor pressure under conditions of cyanogen treatment of carbonic anhydrase.

Isoelectric focusing. The ampholine mixture, pH 3.5 to 10, was used on 5% polyacrylamide gel.

Results

The Michaelis constants shown in Table 1 and 2 respectively represent the results of esterase assays and hydratase assays for the modified and unmodified carbonic anhydrases. In all cases, the modification caused by reaction with cyanogen is irreversible and

Table 1. Esterase Parameters by the p-Nitrophenyl Acetate Assay

Carbonic Anhydrase[a]	E_o(M)	V_{max}(M/min)[b]	V_{max}/E_o	K_m[b]
BCA II	1.3×10^{-6}	8.2×10^{-4}	631	3.1×10^{-2}
CBCA II	1.3×10^{-6}	2.5×10^{-5}	19	2.0×10^{-3}
RCA	9.2×10^{-7}	9.4×10^{-6}	10	2.1×10^{-3}
CRCA	9.2×10^{-7}	9.6×10^{-6}	10	2.5×10^{-3}
DCA	8.9×10^{-7}	4.7×10^{-6}	5	1.3×10^{-3}
CDCA	8.9×10^{-7}	2.9×10^{-6}	3	1.6×10^{-3}
HCAI	8.5×10^{-7}	2.9×10^{-5}	34	2.4×10^{-3}
CHCAI	8.5×10^{-7}	1.3×10^{-6}	2	5.0×10^{-3}

a BCA = bovine carbonic anhydrase II; CBCA = cyanogen treated BCA II; RCA = rabbit CA; CRCA = cyanogen treated RCA; DCA = dog CA; CDCA = cyanogen treated DCA; HCAI = human carbonic anhydrase I; CHCAI = cyanogen treated HCAI.

b Data from Lineweaver-Burke analysis substrate concentrations vary 2×10^{-5}M - 9×10^{-4}M approximately four substrate concentrations for each V_{max} and Km. Each set of assays was performed three times except DCA and CDCA which were only done twice.

Table 2. Hydratase Parameters by Carbon Dioxide Hydration Assay

Carbonic Anhydrase[a]	E_o(M)	V_{max}(M/min)[b]	V_{max}/E_o	K_m[b]
BCA[a] II	2.6×10^{-7}	4.8×10^{-5}	185	2.2×10^{-2}
CBCA II	2.6×10^{-7}	4.8×10^{-5}	185	2.2×10^{-2}
RCA	1.8×10^{-7}	4.5×10^{-6}	25	2.7×10^{-2}
CRCA	1.8×10^{-7}	6.1×10^{-6}	34	4.0×10^{-2}
DCA	1.7×10^{-7}	2.3×10^{-6}	14	3.0×10^{-3}
CDCA	1.7×10^{-7}	6.7×10^{-7}	4	8.8×10^{-4}
HCAI	1.7×10^{-7}	1.4×10^{-5}	82	7.0×10^{-2}
CHCAI	1.7×10^{-7}	2.7×10^{-6}	16	6.0×10^{-2}

a See footnote (a), Table 1.

b Data from Lineweaver-Burke analysis substrate concentrations 1.65×10^{-2}M, serial dilutions of 1.32×10^{-2}M, 8.25×10^{-3}M saturated CO_2/H_2O. Each assay at the lower substrate concentrations was performed twice with the BCA, CBCA 4 times each. At CO_2 = 1.65×10^{-2}M all were done 3 times. The MOPS buffer was 50 mM; the p-nitrophenol indicator was 1.1×10^{-4}M. An Aminco stop flow apparatus was used. The oscilloscope images were photographed and rates at time zero were calculated from these traces.

stable for a period of weeks under conditions of treatment. The control bovine carbonic anhydrase II K_m for p-nitrophenyl acetate hydrolysis is 3.1×10^{-2} and the K_m for carbon dioxide hydration is 2.2×10^{-2}. These correspond well with the published K_m of 1.22×10^{-2} for p-nitrophenyl acetate hydrolysis and the $K_m = 1.55 \times 10^{-2}$ for the carbon dioxide hydration (8). A value given for carbon dioxide hydration by human carbonic anhydrase I(B) is 4 mM (10) which corresponds with the 7 mM obtained in this study.

The results show a specificity of inhibition of the various mammalian carbonic anhydrases. Bovine carbonic anhydrase is clearly inhibited in esterase activity yet shows no change in hydratase activity; V_{max} and K_m are unchanged in the hydratase activity while V_{max} for the p-nitrophenyl acetate esterase assay was reduced to 3% by cyanogen treatment. K_m is decreased to 6.5% for the esterase. Rabbit carbonic anhydrase shows no significant inhibition; values for treated and untreated enzyme are essentially within experimental error. Dog carbonic anhydrase shows some esterase inhibition with a much larger hydratase inhibition. The esterase V_{max} was reduced to 62% and K_m did not change significantly. The dog hydratase activity showed a V_{max} and K_m reduction to 29%. The human esterase showed a reduction in V_{max} to 4.5% and the K_m doubled; with the hydratase activity V_{max} down to 19% and K_m essentially unchanged.

Several irreversible modifications of amino acids in the active site of carbonic anhydrase have been studied (16,17). All have shown inhibition of both hydratase and esterase activities. The four carbonic anhydrases studied here show widely different specificities of inhibition by cyanogen. All but the rabbit carbonic anhydrase are irreversibly inhibited in at least one of the activities.

The cyanogen treated bovine enzyme showed no regain of activity immediately after removing the C_2N_2 by entrainment nor after being stored at ~4° for 72 h. Control enzyme showed no change in activity.

Isoelectric focusing of the cyanogen-treated and untreated bovine enzyme indicated nearly identical pIs; by contrast, proteins treated with 100-fold higher concentrations of C_2N_2 show markedly altered pI values (Tharp and Day, unpublished).

The bovine enzyme esterase activity was not protected from C_2N_2 inhibition by the completely inhibiting concentration of p-aminomethylbenzenesulfonamide, viz., 1.9×10^{4} M. Nevertheless, the C_2N_2 treated enzyme would not bind to an affinity column

containing the bound p-aminomethylbenzenesulfonamide ligand; the unmodified enzyme, of course, did bind. The latter fact was exploited to purify inhibited enzyme.

Discussion

Cyanogen is shown here to irreversibly modify and inhibit three of the carbonic anhydrases studied. The difference in the inhibition of the two activities in the various enzymes may be exploited to gain further understanding of the active site mechanism.

Where affinity labels have been used (8) the various mammalian carbonic anhydrases show characteristic patterns; some of these percentage inhibition values are tabulated with our V_{max} data (Tables 1 & 2) and shown in Table 3. Several effective affinity labels prior to this work are analogs of HCO_3^- and some compete with it during their first, reversible interaction. Other affinity labels are not obvious analogs and their binding appears to be adventitious. However close an analog a given inhibitor may or may not be,

Table 3

Irreversible Inhibition by Active-Site-Directed Reagents

Carbonic Anhydrase	Inhibitor	Molarity	Time(hr.)	% Remaining Activity		Reference
				Hydratase Activity	Esterase Activity	
Human I	Iodoacetamide	25 mM	16	3	30	(20)
Human I	Bromoacetate	24 mM		-[a]	15	(17)
Human I	Iodoacetate	22 mM	2	5	7	(16)
Human II	Bromoacetazolamide	24 μmoles[b]	24	-[a]	2.3	(21)
Bovine	Bromoacetazolamide	24 μmoles[b]	24	-[a]	5	(21)
Human I	Bromopyruvate	3 mM	1	-	4	(22)
Human II	Bromopyruvate	25 mM	>1	20-25	30	(22)
Bovine	Iodoacetate	10 mM	>24	-[a]	0	(23)
Human I[c]	Cyanogen	1 mM	2	19	45	This work
Rabbit[d]	Cyanogen	1 mM	2	135	102	This work
Canine[d]	Cyanogen	1 mM	2	29	62	This work
Bovine II[e]	Cyanogen	1 mM	2	100	3	This work

(a) Activity measurement not reported. (b) Volume not reported. (c) Erythrocyte enzyme, specific isozyme B. (d) Erythrocyte enzyme, specific isozyme not designated.
(e) Erythrocyte enzyme, specific isozyme B.

there appears to be a unique pattern of altering kinetic parameters and sites of binding among the carbonic anhydrases typically examined. Until we have identified binding sites, and done further competition studies, activity/pH profiles, isotope effects, etc., on the modified enzymes, we cannot address the questions of mechanism raised by Silverman and Vincent (18) or Brown and Koenig (19).

It is well known that two hydrolysis products of C_2N_2, HCN and HOCN, are inhibitors of carbonic anhydrase with $K_I = 2.6 \times 10^{-6}$ and 1.1×10^{-4} respectively (14); we ruled out their presence by head space monitoring by mass spectrometry. Further, we showed that HCN inhibition was readily reversed by our entrainment procedure. Appreciable recovery of any lost activity was never seen after removal of C_2N_2 for periods up to 72 h. On the other hand, HCN inhibition was readily reversed by our entrainment process. This implies that covalent modification has occurred, the nature of this is being determined currently; it may involve monofunctional or bifunctional modifications of amine or carboxyl groups according to known chemistry of C_2N_2 (1). Although cyanogen is likely to react with sulfhydryl groups in these enzymes, they are not present.

In this initial report of the first inhibition of carbonic anhydrase by the close structural analog of CO_2, C_2N_2, we have shown that cyanogen is a powerful inhibitor of some carbonic anhydrases.

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