Interaction of Acetazolamide and 4-Nitrothiophenolate Ion with Bivalent Metal Ion Derivatives of Bovine Carbonic Anhydrase†

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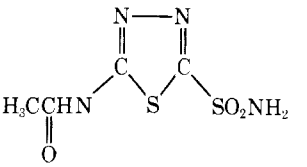
ABSTRACT: The stability and rate constants for the interaction of acetazolamide (diamox) and 4-nitrothiophenolate ion (NTP) with the bivalent Mn, Co, Ni, Cu, and Cd forms of bovine carbonic anhydrase have been measured by utilizing the distinct visible spectra of each metalloenzyme-NTP adduct. Differing stabilities of the various NTP and (particularly) diamox complexes reside mainly in varying values for the dissociation rate constants (k_d). Intrinsic formation rate constants (for the acid form of the enzyme reacting with the basic form of the ligand) are uniformly high ($\geq 2 \times 10^7$ M^{-1} s^{-1} at 25 °C). Invariance of k_d with pH and a bell-shaped log K-pH profile with the Cu-enzyme adducts are features observed previously with the native enzyme. Binding of NTP with the Cu and Cd metalloenzymes is stronger than to the native form.

Carbonic anhydrase (EC 4.2.1.1) is a metalloenzyme containing 1 g-atom of very tightly bound zinc per molecule (Lindskog et al., 1971; Prince and Woolley, 1972; Coleman, 1973; Dunn, 1975). In addition to catalyzing the reversible hydration of CO_2, carbonic anhydrase has been found to catalyze the hydration of certain esters and aldehydes. Aromatic and heterocyclic sulfonamides, as well as a number of anions, are inhibitors of the catalytic action of carbonic anhydrase. There have been a large number of studies of the equilibrium and rate characteristics of inhibitor interaction with carbonic anhydrase, and these have played an important role in defining the nature of the active site of the enzyme (Lindskog et al., 1971; Coleman, 1973; Dunn, 1975).

The zinc ion in carbonic anhydrase can be replaced by a sizeable number of bivalent metal ions (Lindskog and Malmstrom, 1962; Lindskog, 1963; Lindskog and Nyman, 1964; Coleman, 1967b). These metalloforms of the protein have been studied using a number of physical methods appropriate to the substituting metal ion. Thus, characteristics such as spectral, CD,[1] NMR, ESR, fluorescence (Agró et al., 1974) and, recently, nuclear quadrupole interaction (Bauer et al., 1976) have been examined, usually with the aim of understanding the behavior of the native form.

Only meager kinetic and thermodynamic data exist for the interaction of sulfonamides and anions with the metalloforms of carbonic anhydrase, other than those of the native and Co(II) forms. Questions to which answers would be interesting include: (a) How stable are the complexes with these derivatives? Does the enhanced activity of the zinc and cobalt forms of carbonic anhydrase always lead to stronger interaction with ligands than the other metalloforms (Prince and Woolley, 1972)? (b) How labile are the metal centers—does the wide range of reactivity of these metal ions with simpler ligands pertain?

We have chosen to study the equilibria and rate constants for the interaction of two strong inhibitors, acetazolamide (diamox), I, and 4-nitrothiophenolate ion (NTP) with the Mn, Co, Ni, Cu, and Cd forms of bovine carbonic anhydrase. Diamox is a much studied representative of the sulfonamide group of inhibitors, and comparative data for the native and Co(II) enzymes are available. Olander and Kaiser (1971) have shown that NTP is a potent inhibitor of bovine carbonic anhydrase action. The interaction of NTP could also be monitored using the strong (visible) spectral differences between free and protein-associated NTP. These spectral features could also be used to study the interaction of diamox with the metalloforms of carbonic anhydrase, by using competition between diamox and NTP for the metalloprotein.



I

Experimental Section

Bovine carbonic anhydrase (Sigma Chemical Co.) was dialyzed against phenanthroline in 0.1 M acetate buffer at pH 5.0–5.2 for about 7 days in order to obtain the apo form and then dialyzed several times against a large volume of the appropriate buffer (Lindskog and Malmstrom, 1962). The specific activity of the apoenzyme was approximately 1–2% of the native form as determined using p-nitrophenyl acetate hydrolysis at 348 nm (Armstrong et al., 1966). The small residual amount of zinc enzyme did not interfere. The concentration of apoenzyme was determined spectrophotometrically at 280 nm, using $\epsilon_{280} = 5.7 \times 10^4$ M^{-1} cm^{-1} (Lindskog and Nyman, 1964) or (more reliably) by forming at pH 6.0 the copper-carbonic anhydrase-iodide ($\epsilon_{355} = 3.2 \times 10^3$ M^{-1} cm^{-1}; $\epsilon_{445} = 2.8 \times 10^3$ M^{-1} cm^{-1}) or -azide [$\epsilon_{400} = 3.3 \times 10^3$ or 2.7×10^3 M^{-1} cm^{-1} (Morpurgo et al., 1975)] adducts. Stock solutions of the metallocarbonic anhydrase were prepared by mixing apocarbonic anhydrase ($\sim 10^{-3}$ M) with metal ion ($\sim$0.90 equiv) leaving 10–15 min for the formation to be complete. A deficiency of metal ion was used since, in the case of Cu(II), for example, it is known to be difficult to remove excess metal ion (Taylor and Coleman, 1971). Diamox was a gift from Lederle Laboratories, and 4-nitrothiophenol (Aldrich

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[1] Abbreviations used: diamox, acetazolamide; NTP, nitrothiophenolate; CD, circular dichroism; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; phen, 1,6-phenanthroline; M(CA), metal-carbonic anhydrase.

TABLE I: Experimentally Determined Apparent Constants at 25 °C and pH 7.5.

	k_f (M^{-1} s^{-1})	k_d (s^{-1})	K_A (M^{-1})
NTP complexes			
Cu(CA)	6.0×10^7	4.0[a,b]	4×10^7 [c]
	1.4×10^7 [b]	3.3[b]	7×10^6 [c,b]
	$\sim 2 \times 10^7$ [i]		
Co(CA)	$\geq 4 \times 10^7$	400[c,d]	4×10^5 [j]
Ni(CA)	$\geq 1.4 \times 10^7$	≥ 350[e]	8×10^4 [j]
Mn(CA)		≥ 350[c,e]	9×10^4 [j]
Cd(CA)	$\geq 3.5 \times 10^7$	0.08[d,f]	$>10^8$ [j]
Zn(CA)			1×10^6 [j]
			6×10^5 [e]
Diamox complexes			
Cu(CA)	2.0×10^6 [i]	19[a,g]	6.3×10^5 [k]
Ni(CA)		≥ 350[h]	4.5×10^4 [h]
Mn(CA)		≥ 350[h]	4×10^4 [h]
Cd(CA)		29[d,h]	$\geq 3 \times 10^5$ [h]

[a] Scavenging with N_3^-. [b] pH 9.0. [c] Competition with diamox. [d] Using expression 10. [e] Competition with Co(CA). [f] Competition with Hg(CA). [g] $k_d = 18$ s^{-1} at pH 10.2 and 26 s^{-1} at pH 6.0. I^- scavenging gives similar results. [h] Competition with NTP. [i] pH 6.0. [j] Spectral, dilution method. [k] At pH 7.8; for values at other pH's, see Figure 2.

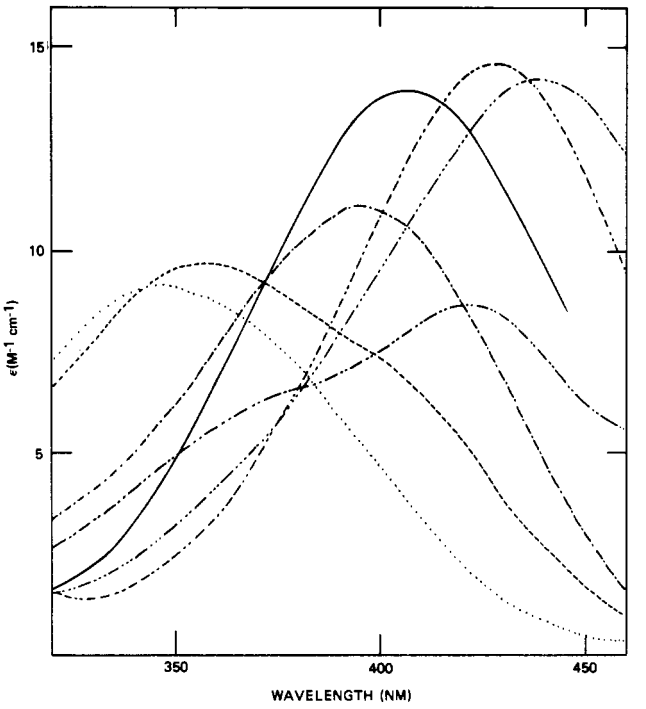

FIGURE 1: Spectra of 4-nitrothiophenolate ion and complexes with metalloderivatives of carbonic anhydrases. (—) NTP; (- - - - - - -) Mn(CA); (- - - -) Co(CA); (- - - - - - - -) Ni(CA); (- - - - -) Cu(CA); (- - - -) Zn(CA); (- - - -) Cd(CA). Left ordinate is $10^{-3} \times \epsilon$.

Chemical Co.) was recrystallized several times from cyclohexane (mp 76–77.5 °C). The buffers used were 2-(*N*-morpholino)ethanesulfonic acid for pH 7, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and tris(hydroxymethyl)-aminomethane (Tris-SO_4 for pH 7.5 and Tris-Cl for pH 9.0). All metal salts were chemically pure; solutions were analyzed by standard methods. All solutions were prepared with water which had been extracted with 0.005% dithizone in CCl_4. Dialysis tubing (VWR Scientific) was prepared by boiling for about 15 min in (a) 1% EDTA, (b) 1% EDTA and 1% Na_2CO_3, and (c) two changes of metal ion free water. All experiments were carried out at 25 °C in 0.01 M buffer. No attempt was made to control ionic strength other than with buffer. It has been observed previously that there is only a small effect of ionic strength on the rate constant for association between human carbonic anhydrase C and *p*-nitrobenzenesulfonamide at pH 7.6 or 9.1 (Taylor et al., 1970b). The rate constants (or lower limits) for the formation of the azide and diamox complexes of Cu–carbonic anhydrase and the NTP complexes of Mn, Ni, Cu, and Cd forms of carbonic anhydrase were determined by mixing as low concentrations of reactants as possible (commensurate with absorbance changes of ~0.02 at 400–440 nm) on a Gibson-Durrum stopped-flow spectrophotometer. Good first-order plots were obtained when 20–50 μM Cu(CA) was mixed with 45–180 μM diamox [X] and 3–30 μM Cu(CA) [X] was mixed with 0.6–5.0 μM NTP. The second-order formation rate constant k_f was calculated from the observed pseudo-first-order rate constant k_{obsd} and the dissociative rate constant (k_d, see below) using the relationship $k_{obsd} = k_f[X] + k_d$. The results are shown in Table I. The reaction rates of Co(II) with apoenzyme were studied as described previously (Gerber et al., 1975).

The rates of dissociation of diamox or NTP complexes were measured in a Gibson-Durrum apparatus at wavelengths in the 350–460-nm region, by using appropriate scavengers for either the released metallocarbonic anhydrase or ligand (Table I). The M(CA)·NTP complex was preformed from equivalent amounts of reactants (5–30 μM). The diamox complex was formed by using 3–20-fold excess of diamox. Except for the Cd(CA)·NTP reaction with Hg(CA), where initial rates were utilized, the scavenging reactions proceeded to ≥95% completion, as evidenced by spectral examination of the products. The conditions and results are collected in Table I.

The spectra of complexes of NTP with metallocarbonic anhydrases were determined at pH 7.5 on mixtures of NTP (~28 μM) with an excess of metalloprotein (~100 μM), sufficient to ensure ≥95% of the protein being associated with the thiophenolate. A correction for the small amount of dissociation could then be made using values of formation constants subsequently determined. The spectra of 4-nitrothiophenolate ion and its Mn, Co, Ni, Cu, Zn, and Cd carbonic anhydrase complexes are shown in Figure 1. The absorption spectra of a number of solutions containing approximately 1:1 ratios of NTP and M–carbonic anhydrase (abbreviated M(CA); M = Mn, Co, Ni, and Zn) in concentrations 10–50 μM were measured at pH 7.5 and, from their absorption coefficients at a number of wavelengths (in the region 340–460 nm), from the known absorption coefficients of the pure complexes, and, by application of material conservation equations, it was possible to calculate an apparent formation constant, K_A = [ligand attached to M(CA)]/[total free M(CA)][total free ligand]. The stability of the Cu(CA) and Cd(CA) complexes with NTP was too high to show perceptible trends in spectral characteristics (e.g., peak positions, intensities) with dilution. Thus, the spectrum of an 80 μM solution of Cu(CA)·NTP or Cd(CA)·NTP was exactly eight times as intense from 340–460 nm, as that of a 10 μM solution. The apparent formation constant of the Cu(CA)·NTP complex was determined at several pH's by spectral measurements of mixtures of known concentrations of Cu(CA)·NTP and diamox, utilizing the separately determined value of K_A for the Cu(CA)·diamox complex. Only a lower limit for the formation constant of the

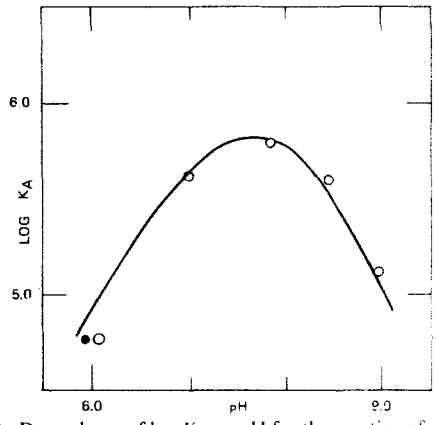

FIGURE 2: Dependence of log K_A on pH for the reaction of copper-bovine carbonic anhydrase with diamox, at 25 °C and [I] = 0.01 M. Open circle values were obtained by the I^- indicator method. The closed circle value was measured directly at 380 nm. The solid curve corresponds to eq 5 using values specified in the text.

Cd(CA)·NTP complex could be assessed since Cd(CA) competed effectively with Cu(CA) for limited amounts of NTP.

The apparent formation constants of the Mn and Ni carbonic anhydrase adducts with diamox at pH 7.5 could be measured by competition of diamox and NTP for the metalloprotein, the distribution of complexes being easily assessed spectrally. The contribution of Mn(CA), Ni(CA), and their diamox complexes is negligible in the 350–450-nm spectral region. The extent of association of diamox with Cd(CA) was assessed from dissociative rate data (using NTP). When a 3.3 μM Cd(CA)–33 μM diamox mixture was treated with 10 μM NTP, ~3.3 μM Cd(CA)·NTP was produced slowly (21 s^{-1}) as shown by the oscilloscope absorption changes. Free Cd(CA) reacts very rapidly with NTP and would not appear as a time-dependent absorption change. The formation constant of Cd(CA)·diamox could therefore be assessed as $\geq 3 \times 10^5$ M^{-1} assuming a $\leq$10% dissociation of the diamox complex.

Finally, the formation constant of Cu(CA) with diamox was determined spectrally at a number of pH's (Figure 2) using iodide ion as an indicator of the concentrations of Cu(CA) in mixtures with diamox. The formation constant of the CuCA·I^- adduct was determined spectrally at pH's 6.1 and 8.5 using the intense absorption of the iodide adduct at 355 and 445 nm ($\epsilon = 3.2 \times 10^3$ and 2.8×10^3 M^{-1} cm^{-1}, respectively) (Morpurgo et al., 1975). The formation constant of Cu(CA)·diamox could also be measured directly at pH 6.0 using the differences in absorption coefficients for Cu(CA)·diamox (ϵ = 540) and Cu(CA) (ϵ = 140) at 380 nm. The value was in excellent agreement with that obtained using iodide ion as an indicator (Figure 2). This direct determination could not be used, however, with any degree of accuracy at other pH's because of the enhanced stability of the diamox complex, and the consequential difficulty of obtaining equilibrium mixtures with spectrally significant amounts of all species. The relative magnitudes of the formation constants of a number of diamox, as well as NTP, complexes were confirmed by competition experiments, using spectral methods. A Cary 14 recording spectrophotometer carefully calibrated with matching cells was used for all spectral measurements.

Results

Equilibrium Constants. The effect of pH on the apparent formation constant has been studied only with the Cu(CA) derivatives. The protein is involved in an acid–base equilibrium centered around the metal site (charges on protein ignored):

$$HCu(CA) \rightleftharpoons Cu(CA) + H^+ \qquad K_E \qquad (1)$$

In the reaction with nonprotic anions I^- and NTP [which with pK_a = 4.5 (Frankfater and Kezdy, 1971) can be considered nonprotic in the range of pH examined, see also below], the adduct HCu(CA)·X (X = I^- or NTP) is formed. The apparent formation constant K_A

$$K_A = \frac{[HCu(CA)\cdot X]}{\{[CuCA] + [HCu(CA)]\}[X]} \qquad (2)$$

and intrinsic formation constant K_I

$$K_I = \frac{[HCu(CA)\cdot X]}{[HCu(CA)][X]} \qquad (3)$$

are related to $[H^+]$ and K_E by the expression:

$$K_A = \frac{K_I[H^+]}{K_E + [H^+]} \qquad (4)$$

Relationship 4 has been verified in detailed studies of the Cu(CA)·I^- (Morpurgo et al., 1975) and Zn(CA)·NTP systems (Olander and Kaiser, 1971). Our (more limited) data for Cu(CA)·I^- at pH 8.5 ($K_A = 6.6 \times 10^3$ M^{-1}) and pH 6.1 ($K_A \sim K_I = 3.2 \times 10^4$) is in reasonable agreement with the literature ($K_I = 1.9 \times 10^4$ M^{-1}). Our data for the Cu(CA)·NTP system also conform to eq 4. On this basis, values of K_I for the reactions of NTP with the Mn, Co, Ni, and Cd metalloforms have been calculated from K_A at pH 7.5 (Table II), using pK_E values of (respectively) 8.2 (Lanir et al., 1975), 6.8 (Lindskog, 1966), 8.0,[2] and 9.1 (Bauer et al., 1976), and are collected in Table II.

The variation of K_A with pH shown in Figure 2 for the reaction of Cu(CA) with diamox can be rationalized as a reaction of the acid form of the carbonic anhydrase with the basic (RSO_2NH^-) form of the diamox.[3] Multiple proton ionizations (from $-SO_2NH_2$ and $-NHCOCH_3$ groups) of the sulfonamide complicate the expression relating K_A and pH (Lindskog, 1969):

$$\frac{1}{K_A} = \left(1 + \frac{K_E}{[H^+]}\right)\left(\frac{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}}{\frac{K_1K_{12}}{K_1} + \frac{K_1'K_2}{[H^+]}}\right) \qquad (5)$$

K_1 and K_2 are the potentiometrically determined ionization constants of diamox (referring to the SO_2NH_2 and $NHCOCH_3$ groups) and K_{12} is the microscopic ionization constant of the $-SO_2NH_2$ group. K_I and K_I' are the formation constants of the $CH_3C(=O)NH\cdot C_2N_2S\cdot SO_2NH^-$ and $CH_3C(=O)N^-\cdot C_2N_2S\cdot SO_2NH^-$ complexed forms of diamox,[3] and these are represented by X in eq 3. The term containing K_I' is relatively unimportant at pH's <9.0. The values used in constructing the solid curve of Figure 2 were $K_I = 2.5 \times 10^6$, $K_I' = 4.1 \times 10^5$, $pK_1 = 7.2$, $pK_2 = 8.8$, $pK_{12} = 7.5$ (Lindskog, 1969), and $pK_E = 8.2$. The experimental data are seen to be in excellent agreement with this calculated curve. Apparent formation constants at pH 7.5 only were determined for di-

[2] A value of K_E for HNi(CA) is apparently not available. It was arbitrarily assigned 8.0. The factor for converting K_A into K_I is quite close to unity, provided $K_E \leq H^+$. The pH value quoted for HCd(CA) refers to the human B form.

[3] The alternative formulation, that of the basic enzyme reacting with nonionized diamox, cannot be ruled out. There is some justification for the reacting partners assumed by us (Dunn, 1975; King and Burgen, 1976).

TABLE II: Intrinsic[a] Stability and Rate Constants at 25 °C for Interaction of M(CA) with NTP and Diamox.

	NTP			Diamox		
M	k_f (M^{-1} s^{-1})	k_d (s^{-1})	K_I (M^{-1})	k_f (M^{-1} s^{-1})	k_d (s^{-1})	K_I (M^{-1})
Cu	5×10^7	4.0	5.0×10^7	5.0×10^7 [c]	20	2.5×10^6
Co	8×10^8 [c]	400	2.0×10^6	7.0×10^7 [d]	0.14[d]	5.0×10^8 [d]
Ni	$\geq 4 \times 10^7$ [c]	≥ 350	1×10^5	$\geq 3.2 \times 10^7$ [c]	≥ 350	9×10^4
Mn	$\geq 5 \times 10^7$ [c]	≥ 350	1×10^5	$\geq 3.2 \times 10^7$ [c]	≥ 350	9.2×10^4
Cd	$\geq 7 \times 10^7$ [c]	0.08	$\gg 10^8$	$\geq 1.5 \times 10^7$ [c]	29	$\geq 5 \times 10^5$
Zn	7×10^8 3.5×10^8 [b]	170[b]	4.3×10^6 2.2×10^6 [b]	8.0×10^7 [e]	0.10[e]	7.0×10^8 [e]

[a] On basis of acidic form of enzyme reacting with ionized form of ligand. [b] Olander and Kaiser, 1971. [c] Calculated from $k_f = k_d K_I$. [d] Lindskog and Thorslund, 1968. [e] Lindskog, 1969.

amox interaction with the Mn, Ni, and Cd metalloproteins (Table I) and were easily converted into K_I values using eq 5 and the appropriate K_E values listed above (Table II). All equilibria data obtained in this study were explainable on the basis of only 1:1 adduct formation between protein and ligand, and competition of ligands for the same site; i.e., ternary complexes are unimportant (Coleman, 1967b; Thorslund and Lindskog, 1967; Whitney, 1970).

Some experiments were carried out with the apo form of carbonic anhydrase in an attempt to diagnose interactions with NTP or diamox. Inclusion of 380 μM apo form in a solution containing 96 μM Ni(CA) and 34 μM NTP at 25 °C and pH 7.5 did not modify the spectrum, and the formation constant of apo·NTP could be estimated as $\leq 5 \times 10^2$ M^{-1}.

If diamox binds near to the metal site in apoenzyme, it was considered that the rate of interaction of apo with metal ion might be retarded over that in the absence of the sulfonamide. Some indication of the formation constant for the apo-diamox adduct might be gleaned from the relative rate constants. It was found surprisingly that diamox enhanced the reentry of Co(II) and Co(phen)$(H_2O)_4^{2+}$ ions. The second-order rate constant for Co^{2+} ion reaction at 25 °C and pH 7.5 was increased from 64 to 217 M^{-1} s^{-1} in the presence of 1.4 mM diamox. The second-order rate constant for Co(phen)$(H_2O)_4^{2+}$ reacting with apo was nearly doubled when 1.0 mM diamox was present.

Rate Constants. The general reaction under consideration is represented by

$$M(CA) + L \leftrightarrows M(CA)L \qquad k_f, k_d, K_A \qquad (6)$$

where in general k_f, k_d, and K_A are pH dependent. The scavenging experiments lead to values for the dissociative rate constants. The scheme can be represented as

$$AB \leftrightarrows A + B \qquad k_1, k_{-1} \qquad (7)$$

$$A + C \rightarrow AC \qquad k_2 \qquad (8)$$

from which, assuming a steady-state concentration of A

$$V = k_{obsd}[AB] = k_2[A][C] = \frac{k_1 k_2 [AB][C]}{k_{-1}[B] + k_2[C]} \qquad (9)$$

In the systems AB = Cu(CA)·NTP, C = N_3^-, and AB = Cu(CA)·diamox, C = N_3^- or I^-, $k_2[C] > k_{-1}[B]$, and $k_{obsd} = k_1$. In these cases the value of k_{obsd} is found to be independent of [C] and gives directly the dissociative rate constant $k_1 = k_d$. The rate constant $k_2 \geq 3.5 \times 10^7$ M^{-1} s^{-1} at pH 6.0 was determined independently. With the Cu(CA)·diamox complex, the value of k_d did not vary from pH 6.5 to pH 10.2; with Cu(CA)·NTP, k_d was similar at pH's 7.5 and 9.0. Acid-catalyzed dissociation may become important at pH 6 since with both complexes k_d is enhanced. With three systems examined (AB = Co(CA)·NTP, C = diamox; AB = Cd(CA)·diamox, C = NTP; and AB = Cd(CA)·NTP, C = Hg(CA)), conditions are obtained in which $k_2[C] \sim k_{-1}[B]$, and these are useful for determining not only k_1 but also the ratio k_2/k_{-1}:

$$\frac{1}{k_{obsd}} = \frac{1}{k_1} + \frac{k_{-1}[B]}{k_1 k_2 [C]} \qquad (10)$$

Plots of k_{obsd}^{-1} vs. $[B][C]^{-1}$ for the systems cited gave values for k_d for Co(CA)·NTP, Cd(CA)·NTP, and Cd(CA)·diamox (Table I) as well as the ratio of rate constants for the formation from the metallocarbonic anhydrase of Co(CA)·NTP and Co(CA)·diamox (11.0) and for the formation of Cd(CA)·NTP and Cd(CA)·diamox (12.5). In a number of other scavenging experiments, the reactions were complete within mixing time and a conservative lower limit (≥ 350 s^{-1}) was assigned to k_d for these complexes (Table I). There was no evidence from our studies for assistance in bond dissociation by the replacing group, a finding similar to that for reaction of human carbonic anhydrase C-*p*-nitrobenzenesulfonamide complex with CN^- (Taylor and Burgen, 1971).

Formation rate constants could be obtained directly by flow methods only for Cu(CA) reacting with NTP and diamox. These were shown to be second order. With other complexes, lower limit values only could be measured (Table I). Since the values of k_d where determined are pH invariant, a situation observed previously (Kernohan, 1966; Lindskog, 1969; Taylor et al., 1970a,b), then the value of k_f, for reaction of M(CA) with NTP or diamox mirrors that of K_A with pH and the intrinsic value of k_f can be calculated from the apparent formation rate constants using relationships analogous to eq 4 and 5, respectively. These values are included in Table II.

Discussion

In the metalloforms of carbonic anhydrase which we have examined, the evidence is sound that the metal is strongly bound to the protein and occupies, or is at least very close to, the zinc site. Thus, none of these metallocarbonic anhydrases bind $^{65}Zn^{2+}$ ion (Coleman, 1965) or have enzyme activity restored by the addition of Zn^{2+} ions (Coleman, 1973). The Mn and Cd forms show distinct activity and inhibition patterns (Smith and Bryant, 1975; Bauer et al., 1976). Finally, high resolution difference electron density maps show no significant

differences in the metal binding sites of the Zn, Co, Cu, and Mn solid forms of human carbonic anhydrase C (Waara, 1974).

It has been previously demonstrated that zinc is not removed from the enzyme by NTP (Olander and Kaiser, 1971). The spectra of the NTP adducts with various metalloproteins shown in Figure 1 are quite distinct from those of the free ligand and ligand solutions containing aquated metal ions. In addition, the adducts of Mn, Co, Ni, and Zn-CA are dissociated by diamox and this confirms that the metal–protein bond has remained intact since diamox reaction with metal ions is very weak. Some changes of spectra with time of Mn(CA)·NTP suggest that dissociation of Mn^{2+} ion may occur in micromolar concentrations of the enzyme adduct (Wilkins and Williams, 1974).

The formation constants of the NTP adducts are uniformly high, at least 10^5 and as high as 10^8 or greater with the Cd derivative. The protein is thus remarkable in enhancing the stability of the metal–S^- bond (just as it is in stabilizing monovalent anion complexes of the Cu, Co, and Zn proteins over the metal ion).[4] This may indicate that bonding at the metal center is reinforced by interaction of the $-C_6H_4NO_2$ moiety with His-63 (models show that this is possible). The high stability of the Zn and Co carbonic anhydrase adducts with sulfonamides is attributed to such reinforcing secondary binding, and for this there is abundant evidence. However, there is no evidence for interaction of NTP with enzyme from which metal ion has been removed, i.e., the apo form. This indicates that the metal bonding to the thiophenolate is a paramount feature of the interaction. We were unable therefore to probe apoenzyme–diamox interaction by using NTP as a monitor. However, it was found that the presence of diamox did markedly enhance the rate of entry of Co(II) complexes into the apo form. Studies from 15 to 35 °C showed that this was not due, however, to large $\Delta H^{\ddagger}$ changes. Although we could not extract any formation constant data for apo–diamox interaction, we feel that binding of diamox occurs near to the metal site and in the process distorts (opens up) this site for easier entry of metal ion.

The obvious bell-shaped relation between K_A and pH shown in Figure 2 for the reaction of Cu(CA) with diamox has been also observed for the zinc- and cobalt–bovine carbonic anhydrases, where a similar interpretation—a reaction of the acid form of the enzyme with the ionized diamox—is proposed (Lindskog and Thorslund, 1968; Lindskog, 1969).

Coleman (1967a) has measured the formation constants at pH 8.0 for reaction of acetazolamide with a number of metalloforms of human carbonic anhydrase B, using [^{3}H]acetazolamide as a monitor. The Zn and Co forms induce the strongest bonding, Mn and Cu are much weaker and, with apo, Ni, Cd, and Hg carbonic anhydrases, binding does not occur until the concentration of diamox has reached 10^{-4} M or greater. From the inhibition of activity of Cd(CA) at pH 9.6, a value for $K_{inhib} \sim 5 \times 10^4$ M^{-1} has been recently obtained for human carbonic anhydrase B (Bauer et al., 1976). Our findings with the bovine form of the enzyme differ somewhat, even allowing for pH and K_E differences in the two studies. The Cu and Cd carbonic anhydrase complexes are quite stable with diamox, and the Mn and Ni forms much less so. None of them approach the stability of the Zn and Co forms, a relative order, however, which does not pertain with the NTP derivatives.

Rate Aspects. The intrinsic rate constants for the formation of NTP and diamox complexes are shown in Table II. They have been measured directly, calculated from the relationship $k_f = K_1 k_d$,[5] or taken from literature inhibition studies. All the values are uniformly high, greater than 2×10^7 M^{-1} s^{-1} and as high as 8×10^8 M^{-1} s^{-1}, which must be approaching a diffusion-controlled value [$\sim 10^9$ M^{-1} s^{-1} (Eigen and Hammes, 1963)]. These high values also require that rate constants estimated on the basis of the un-ionized thiol reacting with the basic form of the enzyme would be much larger, and clearly impossible (Olander and Kaiser, 1971). Although the varying stability constants of human carbonic anhydrase C with sulfonamides appear to reside mainly in varying values of k_f (Taylor et al., 1970a,b; Dunn, 1975; King and Burgen, 1976), it is the value of k_d which controls the different stabilities of metallocarbonic anhydrases with NTP, diamox, and other ligands (see also Lanir and Navon, 1972; Henkens and Sturtevant, 1972). This is well illustrated in the present study by a comparison of the native, Co and Cu forms of the enzyme combining with diamox. The 200–300-fold increase in stability of the Zn and Co adducts over the Cu derivative is contained almost exclusively in a lowered value for k_d (Table II). The high stability of Cd(CA)·NTP, as another example, results from a long half-life, 8.7 s, for its dissociation. It is interesting to note that the marked reduction in stability of the 4-nitrobenzenesulfonamide adduct with carboxymethylated human carbonic anhydrase B compared with that of the native enzyme also resides primarily in an increased dissociation rate (Taylor et al., 1970b).

There are two main centers of the protein with which diamox interacts. One is at the metal center and is associated with the sulfonamide residue [in the anionic form (SO_2NH^-), see, for example, Kumar et al. (1976)]. The heterocyclic portion of the diamox probably binds to a nonpolar region adjacent to the metal (Waara et al., 1973). Formation of the diamox complex can therefore be considered stepwise (Lindskog, 1969; Taylor et al., 1970a,b; Lanir and Navon, 1972; Olander et al., 1973; King and Burgen, 1976):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES \qquad (11)$$

The intermediate ES*, in which the sulfonamide is attached to one site only, is considered to be in very small concentration (King and Burgen, 1976) and therefore

$$k_f = \frac{k_1 k_2}{k_{-1} + k_2};\ k_d = \frac{k_{-1} k_{-2}}{k_{-1} + k_2} \qquad (12)$$

The constancy of k_f for reaction of diamox with the six metalloderivatives tends to rule out interaction at the metal center as being the first step since k_1 and k_1/k_{-1} might be expected to differ with the different metal forms. However, this expectation should be considered carefully since the marked lability of the metal center (see Gerber et al., 1974) may destroy the pattern of a wide range of reactivity observed with simpler complexing by these metal ions (Wilkins, 1974). In addition, the geometry about the metal in solution is uncertain,

[4] The peak of NTP at 409 nm is shifted by Cd^{2+} ion to 365 nm. The stability constant is estimated as $\sim 6 \times 10^3$ M^{-1} at pH 7.5, four or more orders of magnitude weaker than that of the Cd(CA) adduct. Addition of Ni^{2+} ion even in millimolar concentrations does not alter the position or intensity of the NTP peak, and the interaction constant is $<10^2$, once again three or more orders of magnitude less than that of Ni(CA)·NTP.

[5] In the one instance where we could test this relationship, namely, with Cu(CA) and diamox at pH 6.0, the measured value for $k_f = 2.0 \times 10^6$ M^{-1} s^{-1} is in good agreement with that estimated from $k_d K_A$ (1.2×10^6 M^{-1} s^{-1}).

APPENDIX: Kinetic Data at 25 °C for Interaction of Metallocarbonic Anhydrases with NTP and Diamox.

Dissociation

M(CA)	Concn (μM)	NTP	Concn (μM)	Scavenger	Concn (mM)	λ (nm)	pH	k_{obsd} (s^{-1})
Cu	25		25	N_3^-	0.25	330	6.0	4.1
	25		25	N_3^-	0.25	420	6.0	3.9
	25		25	N_3^-	2.5	420	6.0	9.9
	25		25	N_3^-	2.5	410, 480	7.5	4.0
	25		25	N_3^-	0.25	410	7.5	4.1
	25		25	N_3^-	2.5	420	9.0	3.3
	25		25	N_3^-	0.83	420	9.0	3.5
Mn	10		10	Co(CA)	0.03	350, 425	7.0	≥350
	30		30	Diamox	1.5	385	7.0	≥350
Ni	5		5.3	Cu(CA)	0.05	430	7.0	≥350
	30		32	Cu(CA)	0.05	430	7.0	≥350
	10		10	Co(CA)	0.03	430	7.0	≥350
Co	25		32	Diamox	0.15	350, 420	7.0	116
	25		32	Diamox	0.30	350, 420	7.0	230
	8		11	Diamox	1.5	350, 420	7.0	347
	25		32	Diamox	1.5	350, 420	7.0	347
	25		25	Diamox	0.4	350, 420	9.0	116
	25		25	Diamox	1.5	350, 420	9.0	290
	6		6	Diamox	1.5	350, 420	9.0	365
Cd	30		25	Hg(CA)	0.25	400	7.5	0.057[a]
	30		25	Hg(CA)	0.125	400	7.5	0.044[a]
		Diamox						
Cd	30		300	NTP	0.06	440, 330	7.5	18
	10		100	NTP	0.06	440	7.5	25
	3.3		33	NTP	0.06	440, 460	7.5	28
	3.3		33	NTP	0.01	460[b]	7.5	21
Cu	90		300	N_3^-	1.5	400	6.0	25
	54		150	N_3^-	1.0	400	6.5	22
	5.6		150	N_3^-	0.5	400	7.5	19
	5.6		100	N_3^-	1.0	400	7.5	19
	45		150	N_3^-	1.0	400	8.8	18
	45		150	N_3^-	0.1	400	8.8	20
	54		1000	N_3^-	1.0	400	10.2	18
	90		300	I^-	15.0	360, 445	6.0	26
Mn	35		350	NTP	0.003	380, 460	7.5	≥350
	35		350	NTP	0.005	380, 460	7.5	≥350
Ni	35		350	NTP	0.03	400, 460	7.5	≥350

Formation

M(CA)	Concn (μM)	Ligand	Concn (μM)	λ (nm)	pH	k_{obsd} (s^{-1})	k_f ($M^{-1}s^{-1}$)
Cu	15	N_3^-	25	400	~6.0	≥350	$\geq 3.5 \times 10^7$
Cu	50	Diamox	180	400	6.0	385[c]	2.6×10^6[f]
Cu	25	Diamox	45	400	6.0	70[d]	1.6×10^6[f]
Cu	40	Diamox	90	400	6.0	140[e]	1.6×10^6[f]
Cu	30	NTP	10	420	6.0	630	$\sim 2 \times 10^7$
Cu	5.0	NTP	0.8	420	7.5	315	6×10^7
Cu	5.0	NTP	0.8	420	7.5	250	5×10^7
Cu	3.3	NTP	0.6	420	9.0	41	1.4×10^7
Cu	10	NTP	1.7	420	9.0	92	1.0×10^7
Cu	30	NTP	5.0	420	9.0	258	9×10^6
Cd	10	NTP	2.5	440	7.5	≥350	$\geq 3.5 \times 10^7$
	5.0	NTP	2.5	440	7.5	≥350	$\geq 7.0 \times 10^7$
Ni	25	NTP	8.0	390, 460	7.0	≥350	$\geq 1.4 \times 10^7$
Co	10	NTP	4.0	420	7.0, 9.0	≥350	$\geq 4.2 \times 10^7$

[a] Initial rate/[CdCA·NTP]. [b] With these conditions, all Cd(CA) appeared associated with diamox from the magnitude of the spectral change. Free Cd(CA) reacts very rapidly with NTP and would not appear on the oscilloscope as an absorbance change. Therefore the formation constant of Cd(CA)·diamox $\geq 3 \times 10^5$ M^{-1} assuming a ≤10% dissociation of the diamox complex. [c] First-order rate constant determined from end of trace when [diamox] = 140 μM. [d] $[Diamox]_{final}$ = 25 μM. [e] $[Diamox]_{final}$ = 60 μM. [f] $k_{obsd} = k_d + k_f[diamox]$.

and reaction with these ligands may be simple addition, without replacement of coordinated water, since NMR studies have indicated that there is no coordinated H_2O in the low pH form of the Co(II) and Mn(II) forms of the enzyme (Koenig and Brown, 1972; Lanir et al., 1973, 1975). MCD experiments indicate a trigonal bipyramidal to tetrahedral metal coordination change on the addition of diamox to Co(CA) at pH 8 (Kaden et al., 1972). Finally, conformational changes within the sulfonamide complexes with Mn, Co, Ni, Cu, and Zn forms of human carbonic anhydrase C have been detected by relaxation studies and these are ascribed to geometry changes at the metal site (Giannini and Sodini, 1975).

Bearing in mind these possible complications, we favor interaction at the nonmetal site as the first step to form ES* (Taylor et al., 1970a,b; Lanir and Navon, 1972; King and Burgen, 1976). This step is likened to that of the apo form reacting with the sulfonamide. Binding constants for the apo form reacting with a number of substituted benzenesulfonamides have been recently reported and range from 8×10^2 to 9×10^3 M^{-1} (King and Burgen, 1976). If k_1 is considered to be a diffusion-controlled rate constant, the first step will be a rapid preequilibrium (since $k_{-1} \gg k_2$), and $k_f = k_1k_2/k_{-1}$ and $k_d = k_{-2}$. Structure–activity relations in the binding of sulfonamides to carbonic anhydrase have been interpreted on this basis (King and Burgen, 1976). Our values of k_f are independent of metal (Table II) and are consistent with this scheme, if k_1/k_{-1} and k_2 are metal independent. The first step might be anticipated to depend only slightly on the metal enzyme, but it is less obvious that the first-order rate constant for completion of the sulfonamide binding to metal

$$(ES^* \xrightarrow{k_2} ES)$$

will be metal independent. The varying values of k_d will reside in the ease of metal–sulfonamide bond cleavage represented by k_{-2}.

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