# Kinetics of Interaction of Cyanate Ion with Cobalt Bovine Carbonic Anhydrase†

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ABSTRACT: The rate of the reaction of the cyanate adduct of cobalt(II) bovine carbonic anhydrase with *p*-toluenesulfonamide was measured at 25° by spectral stopped-flow analysis. The first-order rate constant for the interconversion was studied as a function of the concentrations of cyanate ion, *p*-toluenesulfonamide, enzyme and buffer as well as pH from 6.0 to 7.5. The kinetic data lead to rate constant values of $4 \pm 2 \times 10^2$ $sec^{-1}$ and $8 \pm 4 \times 10^6$ $M^{-1}$ $sec^{-1}$ for the dissociation and formation of the cyanate adduct.

Carbonic anhydrase (EC 4.2.1.1) is a metalloenzyme containing 1 g-atom of very tightly bound bivalent zinc per molecule (Lindskog *et al.*, 1971; Prince and Woolley, 1972). Replacement of Zn(II) in native carbonic anhydrase by a number of bivalent metal ions can be achieved *via* the apoenzyme, but only the Co(II) derivative approaches the catalytic activity of the native enzyme (Lindskog and Malmstrom, 1962). There is an ionizing group associated with the enzyme with a $pK_a \sim 7$ which is involved in the catalysis.

Aromatic and heterocyclic sulfonamides containing the $-SO_2NH_2$ residue, as well as a large number of univalent anions, inhibit the enzyme action of both native and cobalt(II) carbonic anhydrase (Maren, 1967; Verpoorte *et al.*, 1967; Pocker and Stone, 1968; Lindskog and Thorslund, 1968; Thorslund and Lindskog, 1968). They achieve this by functioning as ligands toward the metal (Lindskog, 1963, 1966; Coleman, 1967, 1968; Riepe and Wang, 1968; Fabry *et al.*, 1970; Ward, 1969, 1970) and in the case of sulfonamides this association is reinforced by interaction of the aromatic ring with a hydrophobic portion of the protein near to the metal (Fridborg *et al.*, 1967; Hower *et al.*, 1971; Lindskog *et al.*, 1971; Liljas *et al.*, 1972).

The kinetics of complex formation between carbonic anhydrase (both Zn and Co holoenzymes) and sulfonamides have been examined using inhibition behavior (Kernohan, 1966; Lindskog and Thorslund, 1968; Lindskog, 1969) and by stopped-flow methods using fluorescence (Taylor *et al.*, 1970a,b) and spectral (Olander and Kaiser, 1970; Olander *et al.*, 1973a,b) monitoring. The bell-shaped profile for the rate constant–pH plot for interaction of sulfonamides with Zn (and Co) carbonic anhydrase can be interpreted in terms of a reaction of either an acidic form of the enzyme with the basic form of the sulfonamide (containing the $-SO_2NH^-$ residue) or a basic form of the enzyme with the neutral sulfonamide. Attempts to distinguish between these possibilities by resorting to plausibility arguments have not been unequivocally successful (Kernohan, 1966; Lindskog and Thorslund, 1968; Lindskog, 1969; Taylor *et al.*, 1970b; Olander and Kaiser, 1970; Olander *et al.*, 1973a,b).

This ambiguity disappears when the association with carbonic anhydrase is examined with anions which are aprotic in the neutral pH region. Unfortunately anion association is very rapid and difficult to measure directly or indirectly from flow experiments (Taylor and Burgen, 1971). Nuclear magnetic resonance line-broadening techniques, however, have been successfully applied to the measurement of the rate constant for reaction of cobalt carbonic anhydrase with a number of carboxylates (Taylor *et al.*, 1971).

The formation constants of carbonic anhydrase with anions vary widely from around unity for fluoride to almost $10^7$ for cyanide ion. There are strong spectral features associated with the cobalt enzyme adducts (Lindskog, 1963, 1966, 1970; Coleman, 1965, 1967, 1968), and we have utilized these, together with stopped-flow analysis, to study the kinetics of reaction of the cobalt carbonic anhydrase–cyanate complex with *p*-toluenesulfonamide to form the sulfonamide adduct. Under favorable circumstances (*vide infra*) these experiments lead to values for the formation and dissociation rate constants for the cyanate complex.

## Methods

Bovine carbonic anhydrase (Sigma Chemical Co.) was chromatographed on a Whatman DE-52 DEAE-cellulose column according to the method of Lindskog (1960). The purity of the leading fraction (termed the B isoenzyme) was checked by gel electrophoresis. The apoenzyme was prepared from this fraction by dialysis against phenanthroline in 0.1 M acetate buffer at pH 5.0–5.2 for about 7 days (Lindskog and Malmstrom, 1962). The cobalt enzyme was prepared by addition of 1.1–1.15 equiv of a $Co^{2+}$ solution to the apoenzyme in a solution containing the appropriate buffer. The specific activity of the apoenzyme was usually approximately 3% of the native form. This small residual amount of zinc form did not interfere with the kinetic experiments since the enzyme was always in deficiency compared with the other reactants. Reagents used were chemically pure. *p*-Toluenesulfonamide was recrystallized several times. The buffers used in the kinetic studies were 2-(*N*-morpholino)ethanesulfonic acid for pH 5.6–6.7, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) for pH 6.8, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid for pH 7.5 (Good *et al.*, 1966). All water, buffer, and ionic strength media were freed from traces of metal ions by shaking with dithizone in carbon tetrachloride.

Stock solutions of $Co^{2+}$ ions were standardized with EDTA using murexide as indicator. Protein concentrations were

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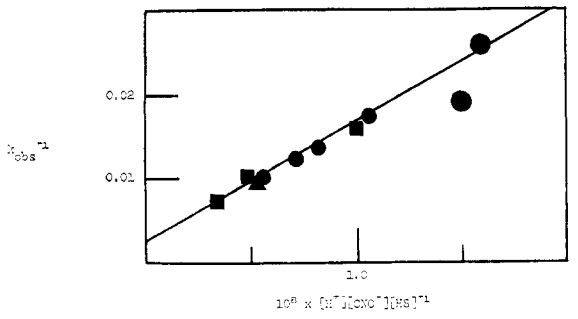


FIGURE 1: Plot of $k_{obsd}^{-1}$ *vs.* $[H^+][CNO^-][HS]^{-1} \times 10^8$ for reaction of cobalt bovine carbonic anhydrase–$CNO^-$ adduct with *p*-toluenesulfonamide at pH 7.5 and 25°: (●) 0.1 M with added KCl; (■) 0.1 M without KCl; (▲) 0.1 M without KCl for pure B isozyme.

determined from absorbances at 280 nm, where $\epsilon = 5.7 \times 10^4$ $M^{-1}$ $cm^{-1}$ (Lindskog and Nyman, 1964). Other solutions were prepared by weight. All runs were carried out at 25° using an ionic strength of 0.1 M. In one set, approximately 0.01 M buffers were used and NaCl was used to make up the ionic strength. In another set, no other anions than those provided by the buffer were present. Most of the experiments used the bovine A + B mixture. Data for the B isoenzyme (Table I and Figures 1 and 2) agreed completely with the mixture. Cobalt bovine carbonic anhydrase A and B have

TABLE I: Kinetic Data for Conversion of Cobalt Bovine Carbonic Anhydrase-Cyanate Adduct to *p*-Toluenesulfonamide (PTS) Complex at 25°.

| [Co Enzyme], mM | [$CNO^-$], mM | [PTS], mM | pH | $k_{obsd}$ $sec^{-1}$ |
|---|---|---|---|---|
| A. $I$ = 0.1 M, with 0.01 M Total Buffer and NaCl | | | | |
| 0.05 | 0.0 | 0.17 | 6.0 | 9.9[a] |
| 0.25 | 2.2 | 8.0 | 6.0 | 3.6 |
| 0.25 | 2.2 | 4.0 | 6.0 | 2.1 |
| 0.20 | 2.5 | 8.5 | 6.7 | 18 |
| 0.20 | 2.3 | 8.5 | 6.8 | 20 |
| 0.20 | 2.5 | 4.4 | 6.8 | 13 |
| 0.07 | 0.0 | 0.17 | 7.5 | 19[b] |
| 0.21 | 1.5 | 8.5 | 7.5 | 100 |
| 0.20 | 2.0 | 8.5 | 7.5 | 80 |
| 0.21 | 3.0 | 8.5 | 7.5 | 57 |
| 0.21 | 4.5 | 8.5 | 7.5 | 39 |
| 0.20 | 2.0 | 4.0 | 7.5 | 53 |
| 0.10 | 1.1 | 4.0 | 7.5 | 74 |
| B. $I$ = 0.1 M, with Approximately 0.1 M Buffer, No $Cl^-$ | | | | |
| 0.04 | 0.25 | 10.0 | 6.0 | 24 |
| 0.075 | 0.47 | 10.0 | 6.0 | 13.5 |
| 0.07 | 1.85 | 10.0 | 6.0 | 3.1 |
| 0.075 | 1.12 | 10.0 | 6.0 | 5.5 |
| 0.075 | 1.12 | 6.7 | 6.0 | 4.1 |
| 0.075 | 1.12 | 3.35 | 6.0 | 2.3 |
| 0.03[c] | 0.60 | 10.0 | 6.0 | 15.3 |
| 0.03[c] | 0.60 | 6.7 | 6.0 | 10.6 |
| 0.075 | 1.12 | 10.0 | 7.5 | 130 |
| 0.075 | 1.12 | 6.7 | 7.5 | 96 |
| 0.075 | 1.12 | 3.35 | 7.5 | 61 |
| 0.03[c] | 0.60 | 3.35 | 7.5 | 102 |

[a] Leads to second-order rate constant $5.8 \times 10^4$ $M^{-1}$ $sec^{-1}$. [b] $1.2 \times 10^5$ $M^{-1}$ $sec^{-1}$. [c] Using separated B isozyme.

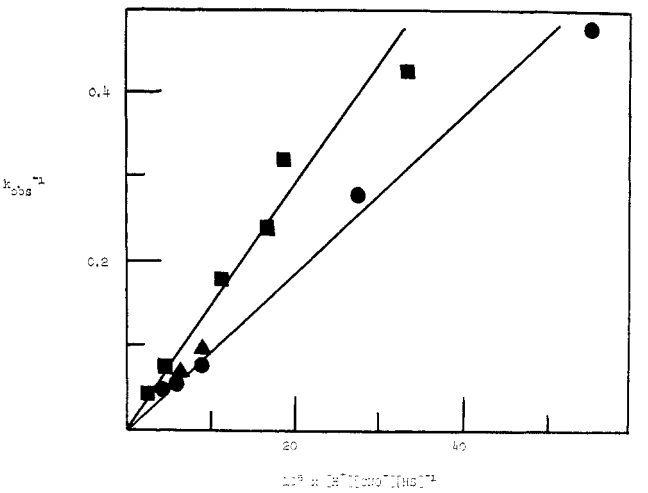


FIGURE 2: Plot of $k_{obsd}^{-1}$ *vs.* $[H^+][CNO^-][HS]^{-1} \times 10^8$ for reaction of cobalt bovine carbonic anhydrase–$CNO^-$ adduct with *p*-toluenesulfonamide at pH 6.0–7.5 and 25°. Symbols as in Figure 1.

identical spectra and p$K$ values (Lindskog, 1963). Cobalt(II) enzyme, cyanate ion, and buffer solution from one syringe was mixed with *p*-toluenesulfonamide and buffer solution from the other syringe in a Gibson-Durrum stopped-flow spectrometer. The decrease in absorbance at 580 nm was monitored on a storage oscilloscope and these traces were photographed. Excellent pseudo-first-order plots (with associated rate constant, $k_{obsd}$) were obtained in the usual way. The results are shown in Table I. In all experiments the cyanate adduct was completely converted to the *p*-toluenesulfonamide complex. The reaction of the cobalt(II) enzyme with *p*-toluenesulfonamide was also investigated at 580 nm in a stopped-flow apparatus (Table IA). Later work used the improved Gibson-Durrum spectrometer and much lower concentrations of enzyme could then be used (Table IB). The formation constant of the cyanate adduct was determined from a series of spectra at different [$CNO^-$] at pH 6, where the acid form of the enzyme predominates.

## Results

We attempted to measure the rate constant directly for the reaction between cobalt carbonic anhydrase and cyanate ion. Using as low concentrations of reactants as possible ($10^{-4}$ M enzyme, $5 \times 10^{-4}$ M $CNO^-$, pH 6.0) which would still promote substantial formation of adduct, the reaction was complete within flow mixing times. This requires that the second-order formation rate constant $\geq 4 \times 10^5$ $M^{-1}$ $sec^{-1}$ at 25°. A similar result was obtained for the analogous reaction with $N_3^-$ ion. The dissociation of the cyanate adduct was then examined by mixing with azide ion to convert it into the azide complex. Again, the reaction was complete within mixing time, so that the first-order dissociation rate constant was $\geq 2 \times 10^2$ $sec^{-1}$ and the associated formation rate constant $\geq 4 \times 10^6$ $M^{-1}$ $sec^{-1}$.[1] It was not expected in the latter experiments that the scavenging (azide) ion would speed up the dissociation of the enzyme adduct, from previous types of observations (Taylor *et al.*, 1970a, 1971; Taylor and Burgen, 1971; Olander and Kaiser, 1971).

[1] The formation rate constant is obtained from the product of the dissociation rate constant and the association constant of the adduct. The latter value was determined spectrally as $2 \pm 0.5 \times 10^4$ M at 25°. This is a lower value than that of $6 \times 10^4$ M obtained by Lindskog (1963) in a different medium.

TABLE II: Rate Constants at 25° for Interaction of Carbonic Anhydrases with Ligands.

| Enzyme | Ligand | $k_f$ $M^{-1}$ $sec^{-1}$ | $k_r$ $sec^{-1}$ | Ref |
|---|---|---|---|---|
| Co(II)human C | $HCOO^-$ | $3.9 \times 10^8$ | $6.0 \times 10^4$ | *a* |
| | $CH_2FCOO^-$ | $2.2 \times 10^8$ | $2.0 \times 10^5$ | *a* |
| | $CHF_2COO^-$ | $1.9 \times 10^8$ | $1.0 \times 10^5$ | *a* |
| | $CF_3COO^-$ | $2.0 \times 10^8$ | $1.5 \times 10^5$ | *a* |
| Co(II)bovine B | $CNO^-$ | $\sim 8 \times 10^6$ | $\sim 4 \times 10^2$ | *b* |
| Zn(II)human C | $CN^-$ | $>3 \times 10^9$ | $>10^2$ | *c* |
| Zn(II)bovine(A + B) | 4-$NO_2$-thiophenolate | $3 \times 10^9$ | $1.7 \times 10^2$ | *d* |
| Zn(II)human C | *p*-$NO_2C_6H_4SO_2NH^-$ | $7.5 \times 10^8$ | $4.9 \times 10^{-2}$ | *e* |
| | *p*-$NO_2C_6H_4SO_2NH_2$ | $1.5 \times 10^6$ [f] | $4.9 \times 10^{-2}$ | *e* |

[a] Taylor *et al.* (1971). [b] This work. [c] Taylor and Burgen (1971). [d] Olander and Kaiser (1971). [e] Taylor *et al.* (1970a,b). [f] Rate constant for reaction of basic form of enzyme.

Rate constants measurable by flow methods were obtained when cobalt carbonic anhydrase–cyanate complex was treated with *p*-toluenesulfonamide at pH 6.0–7.5. Although the sulfonamide is not a very effective kinetic scavenger for the enzyme resulting from dissociation of the cyanate adduct, it is capable of completely converting the enzyme to the sulfonamide complex. The competitive situation prevails therefore where the overall rate constant is easily measurable although the individual rate constants may not be. The rate constant for conversion of the cyanate to the *p*-toluenesulfonamide complex was studied as a function of the concentrations of $CNO^-$, *p*-toluenesulfonamide, enzyme and pH as well as that of buffer and $Cl^-$. The concentrations of the reactants, $CNO^-$, *p*-toluenesulfonamide, and $H^+$ remain constant in a particular run since much lower concentrations of enzyme are used (see Table I). The reactions which are of concern are

$$HCA \cdot CNO \rightleftharpoons HCA^+ + CNO^- \qquad k_1, k_{-1}, K_1 \qquad (1)$$

$$HCA^+ \rightleftharpoons CA + H^+ \qquad k_2, k_{-2}, K_2 \qquad (2)$$

$$HS \rightleftharpoons H^+ + S^- \qquad k_3, k_{-3}, K_3 \qquad (3)$$

$$CA + HS \longrightarrow CA \cdot HS \qquad k_4 \qquad (4)$$

$$HCA^+ + S^- \longrightarrow CA \cdot HS \qquad k_4' \qquad (5)$$

The cyanate ion is considered to combine with the acid form of the enzyme (which is represented as $HCA^+$) without any proton release, and the dissociation rate constant for the cyanate adduct is assumed to be pH independent. There is ample justification for these assumptions. The complex HCA·CNO is the predominant species at pH 6.0–7.5, and only forms appreciable amounts of CA at pH $\geq 8$ (Lindskog, 1963). The rate constant for dissociation of human carbonic anhydrase C–formate is nearly pH independent from pH 6.3 to 9.5 (Taylor *et al.*, 1971). Finally, it is the acid form of carbonic anhydrase which reacts with 4-nitro- and 2,4-dinitrophenolate anion rather than the reverse (Olander and Kaiser, 1971).

The final step may be reaction (4) of the basic form of the enzyme (CA), arising from deprotonation of the acid form, with the neutral sulfonamide (HS) which is the predominant form in the pH range we investigated. Alternatively the product may result from (5), a reaction of the acid form of the enzyme with the basic sulfonamide form $S^-$ (containing the $-SO_2NH^-$ residue). The likelihood of (4) or (5) is not resolved in this study; nor is it important in our analysis. In the *p*-toluenesulfonamide complex, the sulfonamide may be present as the anionic form, by internal transfer of a proton (King and Burgen, 1970; Taylor *et al.*, 1970b). Dissociation of CA·HS is negligible with the conditions of the experiments (Taylor *et al.*, 1970a,b).

In understanding the kinetic data for conversion of cyanate to *p*-toluenesulfonamide complex, the simplest assumption is that equilibria (1), (2), and (3) are established rapidly and that the rate-determining step is either (4) or (5). In these cases, the value of $k_{obsd}$, the first-order rate constant for loss of cyanate adduct, is given by

$$k_{obsd} = \frac{k_4 K_1 K_2 [HS]}{[H^+][CNO^-]} = \frac{k_4' K_1 K_3 [HS]}{[H^+][CNO^-]} \qquad (6)$$

These are equivalent since $k_4' = k_4 K_2 / K_3$ (Taylor *et al.*, 1970b). From (6)

$$\frac{1}{k_{obsd}} = \frac{[H^+][CNO^-]}{k_4 K_1 K_2 [HS]} = \frac{[H^+][CNO^-]}{k_4' K_1 K_3 [HS]} \qquad (7)$$

A plot of $k_{obsd}^{-1}$ *vs.* $[H^+][CNO^-]/[HS]$ should therefore be linear and intercept at the origin of the axes. Such a plot is made in Figure 1 for two series of experiments both at pH 7.5 and $I = 0.1$ M, with and without $Cl^-$ ion. These data indicate, particularly for the latter series, a definite intercept for the linear plot. An additional term should therefore be included in (7) to accurately reproduce the data of Figure 1. This can be accomplished by assuming that only equilibria (2) and (3) are established rapidly. Now it is easily shown that

$$\frac{1}{k_{obsd}} = \frac{1}{k_1} + \frac{[H^+][CNO^-]}{k_4 K_1 K_2 [HS]} \qquad (8)$$

(and with a corresponding expression involving $k_4'$). The value of the intercept of Figure 1 is therefore $k_1^{-1}$. Because the observed rate constants are high at pH 7.5, there is a relatively large error in their values and therefore in the intercept. From Figure 1 (and combining the two series of experiments) values of $k_1 = 4 \pm 2 \times 10^2$ $sec^{-1}$ and $k_{-1} = 8 \pm 4 \times 10^6$ $M^{-1}$ $sec^{-1}$ are obtained, both consistent with the qualitative results described at the beginning of this section. We concede that the intercept might be nearer zero than is shown in which case $k_1 \geq 10^3$ $sec^{-1}$ and $k_{-1} \geq 10^7$ $M^{-1}$ $sec^{-1}$.

On either the basis of (7) or (8) the value of the slope of Figure 1 is $(k_4 K_1 K_2)^{-1}$. In order to get a more accurate value for this, a wider range of ordinate values were examined by studying the reaction at lower pH's. The results are shown in Figure 2. In the absence of $Cl^-$ ion, $K_1 = 5 \times 10^{-5}$ M, $K_2 = 1.6 \times 10^{-7}$ M (Lindskog, 1970), and the slope $= 1.5 \times 10^6$ $M^{-1}$ sec. This leads to a value of $k_4 = 8.3 \times 10^4$ $M^{-1}$ $sec^{-1}$. In the presence of 0.1 M $Cl^-$, $K_2 = 5 \times 10^{-8}$ M (Lindskog,

1970) and with a slope = $1.0 \times 10^6$ $M^{-1}$ sec, $k_4 = 4 \times 10^5$ $M^{-1}$ $sec^{-1}$. This is quite close to the experimentally determined value of $k_4$ in 0.1 M $Cl^-$ of $3 \times 10^5$ $M^{-1}$ $sec^{-1}$.[2] Since buffer participation is invoked in the very rapid active-site ionization associated with (2) (Khalifah, 1973; Lindskog and Coleman, 1973), the absence of an appreciable effect of a tenfold buffer concentration changes on our results (Figures 1 and 2) confirms that a rate-determining proton transfer should not be invoked in our scheme. A previous treatment which led to a determination of rate constants for (2) appears now not to be warranted (Wilkins, 1973). We conclude then that (1) and (4) (or (5)) contribute to the overall rate of the interchange, and that the proton–base equilibria (2) and (3) are rapidly established.

Reaction rate constants for enzyme reaction with a number of anions are shown in Table II. The values are all large and are comparable to those for substitution in simpler cobalt(II) tetrahedral complexes in aqueous solution for which nmr exchange rate constants are in the range $10^7$–$10^8$ $sec^{-1}$ (Zeltmann *et al.*, 1969; Zeltmann and Morgan, 1970). These large rate constants may also be associated with a unique geometry for the metal coordination (Taylor *et al.*, 1971) or may arise from geometrical changes that appear to occur when inhibitor binds to cobalt carbonic anhydrase in basic solution (Lindskog, 1963; Kaden *et al.*, 1972). The reactions may thus be addition rather than substitution processes.

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[2] This value for the intrinsic rate constant for reaction between the basic form of the enzyme and neutral sulfonamide ($k_4$) is obtained from the $k_{obsd}$ value at pH 7.5 in the absence of $CNO^-$ ion (Table I). The value of $k_4$ is given by $k_{obsd}(1 + K_3[H^+]^{-1})(1 + [H^+]K_2^{-1})$ and the values $K_2 = 10^{-7.3}$ and $K_3 = 10^{-10.2}$ have been used for determining $k_4$ as $4 \times 10^5$ $M^{-1}$ $sec^{-1}$. The value for $k_4'$ equals $k_4K_2/K_3 = 3 \times 10^8$ $M^{-1}$ $sec^{-1}$ (Taylor *et al.*, 1970b). From data for the interaction of *p*-toluenesulfonamide with human carbonic anhydrases at pH 6.5 (Taylor *et al.*, 1970a) the corresponding values for $k_4$ and $k_4'$ can be estimated as $2.7 \times 10^5$ $M^{-1}$ $sec^{-1}$ and $1.4 \times 10^8$ $M^{-1}$ $sec^{-1}$ quite close to our values for the bovine–Co form of the enzyme.