Interaction of Cobalt(II)–Carbonic Anhydrase with Anions*

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ABSTRACT: The effects of a number of anions on the enzymic activity and the visible absorption spectrum of bovine Co(II)–carbonic anhydrase have been studied in the pH region 6–9. The catalytic rate of hydration of carbon dioxide depends on pH as if the unprotonated form of a group ionizing in this pH range were required for activity. The observed anionic inhibition can be explained as due to specific binding of an ion to a site closely linked to this group so that the binding strength decreases by a factor of approximately 50 when a proton is dissociated from the group. The inhibitory powers of the ions investigated increase in the following order: $F^- < Cl^- < Br^- \ll I^- < NO_3^- \ll NCO^-$. The pH-dependent change of the visible absorption spectrum of the enzyme is shifted to more alkaline pH values in the presence of anions. Each enzyme–anion complex shows its characteristic spectrum with absorption maxima in the region 470–600 mμ. The form of the enzyme which catalyzes the hydration of CO_2 seems to be represented by a spectral species which, in addition to an absorption maximum at 550 mμ, shows a characteristic double peak at 618–640 mμ.

The zinc ion in native carbonic anhydrase can be removed and a catalytically active Co(II)–enzyme can be made (Lindskog and Malmström, 1962). This metalloenzyme is colored with an absorption maximum at 550 mμ, and its spectrum undergoes a marked change in the pH region 6–8 with the appearance in alkaline solutions of two additional maxima at 618 and 640 mμ. When an inhibitor, such as cyanide, sulfide, or a sulfonamide, is added, a new spectrum is obtained but at sufficiently high pH values the characteristic "alkaline" spectrum appears again (Lindskog, 1963; Lindskog and Nyman, 1964). The tentative explanation of these data was that one ligand group could be displaced from the metal ion either by binding a proton or by the inhibitor molecule occupying its coordination site on the metal ion. It was thus predicted that the inhibitory power of these substances should disappear at alkaline pH values. The observation by Roughton and Booth (1946), that the inhibitory effect of Cl^- was abolished above pH 8.6, seemed to support the idea of a correlation between enzymic activity and spectral changes. Recently, Kernohan (1964, 1965) investigated in detail the effects of pH and of Cl^- and NO_3^- on the activity of bovine carbonic anhydrase. He found that one ionizing group is involved in the catalysis, the unprotonated form of which is active in the hydration reaction and the protonated form in the reverse reaction with HCO_3^- as substrate. Dissociation of a proton from this group resulted in a greatly reduced but not abolished binding of the inhibiting anion. In this paper, the absorption spectrum of Co(II)–carbonic anhydrase as an environment-sensitive "label" at the active site is further utilized through a combination of kinetic and spectrophotometric experiments to obtain information about the interaction of the enzyme with substrate, protons, and anionic inhibitors.

Materials

Bovine carbonic anhydrase B was purified from red blood cells according to the method of Lindskog (1960). Metal-free enzyme was prepared by dialyzing the native enzyme against 0.01 M 1,10-phenanthroline in 0.1 M acetate buffer, pH 5.2, for 7 days (Lindskog and Malmström, 1962). The chelator was removed by dialysis against frequent changes of water. The specific activity of the enzyme was then 2.7% of that of the native enzyme and this residual activity was assumed to be due to incomplete removal of Zn^{2+}. Co(II)–carbonic anhydrase[1] was obtained by addition of 1 equiv of $CoCl_2$ to the solution of metal-free enzyme and subsequently adjusting the pH to 7 with 1 M NaOH (Lindskog, 1963). A precipitate of denatured material was centrifuged off.

Glass-distilled water was used throughout the investigation. The water was free from heavy metal impurities as tested by shaking with a 0.01% solution of dithizone in CCl_4. Analytical grade chemicals were used without purification. *p*-Nitrophenol (Fisher Scientific Co.) was recrystallized once from water.

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[1] In this and previous papers (Lindskog, 1963; Lindskog and Nyman, 1964) the metal ion in the cobalt–apoenzyme complex has been assumed to be divalent. The results of recent measurements of magnetic susceptibility (A. Ehrenberg and S. Lindskog, unpublished data) show that the oxidation state of the cobalt ion *in situ* is, indeed, +2.

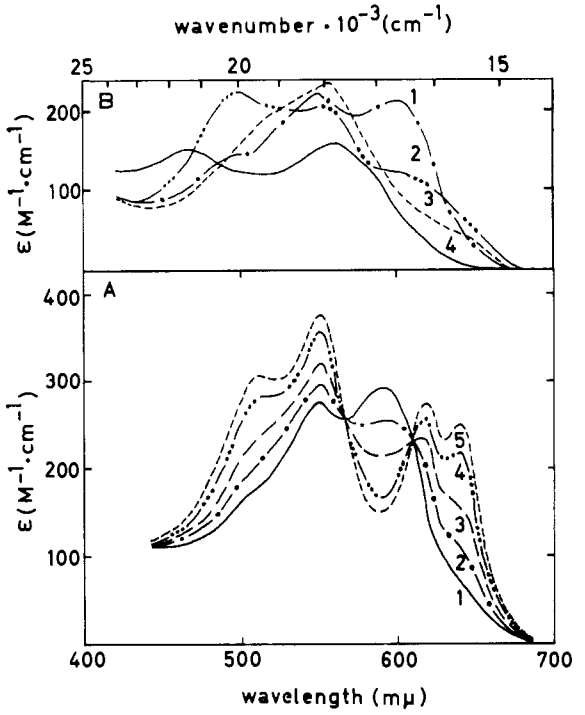

FIGURE 1: Absorption spectra of Co(II)–carbonic anhydrase in 0.025 M imidazole buffers containing 0.1 M Cl^- as the only anionic species (A) and containing only one anionic species (B). A: Temperature, 25°; enzyme concentration, 7.75 × 10^{-5} M. (———) pH 6.29; (—·—) pH 6.66; (—— ——) pH 7.06; (—· · · ·—) pH 7.51; (- - - -) pH 7.90. B: Temperature, 25°; enzyme concentrations, 5.8–6.5 × 10^{-5} M. (1), 0.1 M Br^-, pH 6.21; (2), 0.1 M I^-, pH 7.39; (3), 0.1 M F^-, pH 6.37; (4), 0.1 M NO_3^-, pH 6.23.

Solutions of CO_2, saturated at atmospheric pressure, were made by bubbling gaseous CO_2 (bone dry, Matheson Co.) through water or a solution of neutral salt.

Methods

The rates of hydration of CO_2 were studied with an indicator method. A CO_2 solution was rapidly mixed in a stopped-flow apparatus (Sturtevant, 1964) with a buffer solution containing enzyme and an indicator. The reaction was followed spectrophotometrically at 400 (imidazole buffers with *p*-nitrophenol) or 560 mμ (triethanolamine buffers with phenol red). All experiments were carried out at 25 ± 0.2°.

Enzyme concentrations were estimated spectrophotometrically at 280 mμ assuming ϵ_{280} 57,000 M^{-1} × cm^{-1} and a molecular weight of 30,000 (Nyman and Lindskog, 1964). Carbon dioxide solutions of different concentrations were made from the saturated stock solution by dilution and were immediately transferred to a storage syringe of the stopped-flow apparatus. Since the handling of these solutions caused some CO_2 gas to escape, they were always analyzed just after filling the syringe. The analytical procedure (Gibbons and Edsall, 1963) involved addition of a known excess of 0.04 M $Ba(OH)_2$ and back-titration with 0.01 M HCl with phenolphthalein as indicator.

The initial rates of CO_2 hydration were calculated according to Gibbons and Edsall (1963) and were corrected for the nonenzymic rate as described by Kernohan (1965). The effect of the back reaction due to HCO_3^- present in the initial CO_2 solutions was estimated to be less than 2% of the total rate and was neglected. The enzymic rates were proportional to enzyme concentration in the range employed. In most of the kinetic experiments the enzyme concentration was chosen to give an apparent reaction half-time of 0.2–1 sec (usually 7 × 10^{-8} M). The correction for the nonenzymic rate was then less than 5% except at the highest pH values when it could amount to 15%. The preparation of Co(II)–enzyme contained 2.7% of Zn(II)–enzyme (see Materials) and a correction was applied to obtain the rate due to Co(II)–enzyme alone. The rate of the Zn(II)–enzyme-catalyzed reaction was measured separately at two different substrate concentrations for each buffer composition. The magnitude of the correction was estimated by extrapolation and was usually less than 10% but in a few cases amounted to 25% of the total enzymic rate.

For each buffer composition at least six different CO_2 concentrations ranging from 1.5 to 15 mM were employed. If such a set of measurements was completed within approximately 3 hr, the enzyme lost no activity as tested by running the highest substrate concentration both at the start and at the end of the experiment. No stabilizing substance, such as peptone, was thus considered necessary (*cf.* Gibbons and Edsall, 1964). The enzyme stock solution (8 × 10^{-4} M) did not loose any significant activity in 6 months. The kinetic parameters, V_M and K_m, were obtained from curves of initial rates, v_0, *vs.* $v_0/(s_0)$, which did not show any consistent deviations from linearity. It is estimated that the uncertainty in these parameters does not exceed 15%. Absorption spectra of the Co(II)–enzyme were measured in a Cary 14 spectrophotometer using cylindrical 5-cm cells.

Results

Effect of pH. The activity and absorption spectrum of Co(II)–carbonic anhydrase were studied as functions of pH at three different Cl^- concentrations, 0.025, 0.100, and 0.500 M, respectively. Corresponding experiments were performed in the presence of 0.100 M concentrations of F^-, Br^-, I^- and NO_3^- and 5 × 10^{-4} M cyanate, respectively. Figure 1A shows the spectra obtained with 0.100 M Cl^-. Similar spectral changes were observed with the other anions. The spectra in the acid pH range are different for the various anions as shown in Figure 1B, whereas the "alkaline" spectrum is always the same. Below pH 8, both the kinetic parameters, $k_2' = V_M/(E_0)$ and K_m, show a considerable variation with pH and anion concentration as illustrated in Figure 2, but in the alkaline region they tend to become pH independent. The results obtained above pH 8.4 for Cl^- and NO_3^-

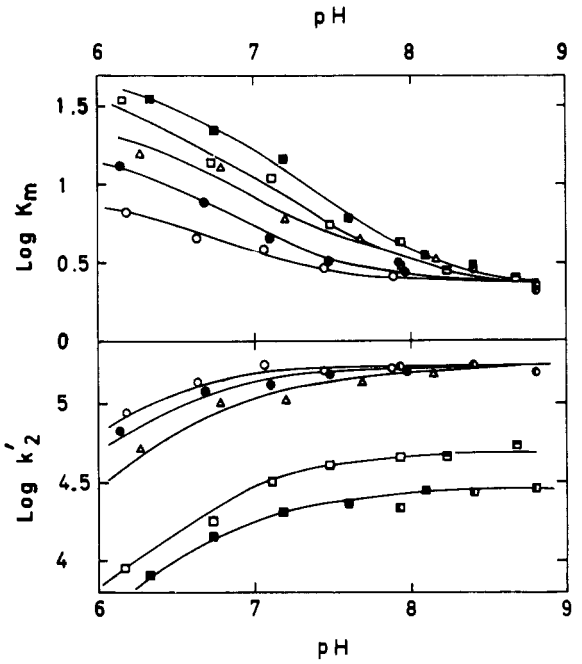

FIGURE 2: Logarithmic diagrams of maximal initial rate constant, $k_2' = V_M/(E_0)$ (sec^{-1}), and apparent Michaelis constant, K_m (mM), as functions of pH. Temperature, 25°; concentration of Co(II)–carbonic anhydrase, 7.2×10^{-8} to 1.58×10^{-6} M. Buffers, 0.025 M imidazole (empty or filled symbols), or 0.025 M triethanolamine (half-filled symbols), containing only one anionic species. Anions: (○), 0.025 M Cl^-; (●,◐), 0.100 M Cl^-; (△), 0.500 M Cl^-; (□,▭), 0.100 M I^-; (■,◧), 0.100 M NO_3^-.

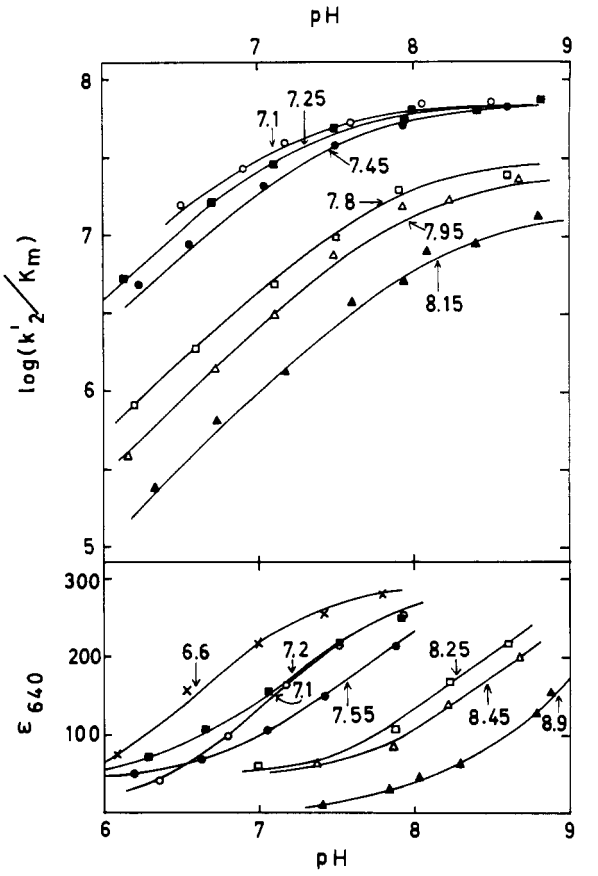

FIGURE 3: The pH dependence of the activity (top diagram) and absorption at 640 mμ (bottom diagram) of Co(II)–carbonic anhydrase. (○), 0.1 M F^-; (■), 0.1 M Cl^-; (●), 0.1 M Br^-; (□), 5×10^{-4} M NCO^- + 0.1 M Cl^-; (△), 0.1 M I^-; (▲), 0.1 M NO_3^-; (×), 0.0333 M SO_4^{-2}. Temperature, 25°. The curves have been drawn to represent titrations of single groups with the values of pK_{app} indicated in the diagrams. Maximal values of log (k_2'/K_m) used were 7.85 (F^-, Cl^-, Br^-), 7.5 (NCO^-), 7.4 (I^-), and 7.15 (NO_3^-), respectively. Maximal value of ϵ_{640} was 300 M^{-1} in all cases. Minimal values of ϵ_{640} were estimated to be 0 (F^-, SO_4^{-2}), 5 (NO_3^-), 40 (Br^-, NCO^-, I^-), and 50 $M^{-1} \times cm^{-1}$ (Cl^-), respectively. See text and legends to Figures 1 and 2 for further details.

suggest that K_m approximates an equilibrium constant, since a sixfold difference in k_2' is not accompanied by any significant difference in K_m. The rate curves were identical if the anion was added to the enzyme syringe or to the substrate syringe before mixing. This suggests that the reaction between enzyme and anion is fast compared to the over-all reaction under the conditions used.

Since the absorption spectra were measured in the absence of substrate, the relevant kinetic parameter is k_2'/K_m, which includes both the competitive and noncompetitive components of the inhibition and reflects the binding of protons and inhibitors to the free enzyme rather than the enzyme–substrate complex (Alberty, 1954; see also Webb, 1963). The substrate and the anions do not bind protons in the pH range investigated. Plots of log (k_2'/K_m) *vs.* pH are shown in Figure 3. Included in the same figure are the corresponding spectral data expressed as the extinction coefficient at 640 mμ, ϵ_{640}. The spectral shift was also studied in the presence of one divalent anion, SO_4^{2-}. The spectrum at approximately pH 6 was identical within the limits of error with that obtained in the experiment with F^- (see spectrum 3 in Figure 1B).

The change from +1 to 0 of the slopes of the logarithmic activity curves in Figure 3 suggests that the binding of one proton to a group in the active site suffices to abolish the CO_2 hydration activity. The activity data are simplest summarized by the equation

$$\frac{k_2'}{K_m} = \frac{k_2}{K_s} \frac{1}{1 + \frac{(I)}{K_B}} \frac{1}{1 + \frac{(H^+)}{K_H}\left[\frac{1 + (I)/K_A}{1 + (I)/K_B}\right]} \quad (1)$$

where k_2 and K_s are the pH- and inhibitor-independent maximal rate constant and Michaelis constant, respectively, and K_H and $K_A < K_B$ are dissociation constants. This would correspond to a scheme where the active

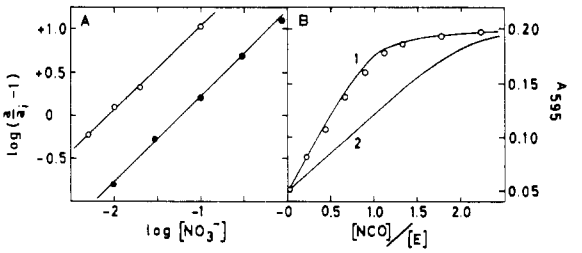

FIGURE 4: Dependence of the activity and absorption at 640 mμ of Co(II)–carbonic anhydrase on the concentration of NO_3^- (A) and spectrophotometric titration of Co(II)–carbonic anhydrase with potassium cyanate (B). A: (○), a and a_i represent activity (k_2'/K_m) at pH 7.95 in the absence and presence of NO_3^-, respectively. The ionic strength was kept at 0.1 with KCl which does not inhibit appreciably at this pH. Temperature, 25°; 0.025 M imidazole buffers. (●), a and a_i represent (ϵ_{640} −5) $M^{-1} \times cm^{-1}$ in triethanolamine buffers, pH 8.8, in the absence and presence of NO_3^-, respectively. B: Aliquots (5 μl) of 0.025 M KNCO were added to 1.5 ml of 3.75×10^{-4} M Co(II)–enzyme in 0.5 M imidazole–H_2SO_4 buffer, pH 6.1. The visible absorption spectrum was measured in a 1-cm cell after each addition of KNCO. The absorption at the wavelength of greatest change (595 mμ; see Lindskog, 1963) is plotted against the molar ratio of total cyanate concentration and total enzyme concentration. Curve 1 is calculated for the binding of only one ion with an apparent dissociation constant of 1.2×10^{-5} M. Curve 2 is calculated for the case with two equally strong binding sites, only one of them being linked to the spectral change.

form of the enzyme, E, becomes inactive by binding a proton or an inhibitor ion or both

$$\begin{array}{ccc} EH + I & \overset{K_H}{\rightleftharpoons} & E + H^+ + I \\ K_A \downarrow\uparrow & & \uparrow\downarrow K_B \\ EHI & \rightleftharpoons & EI + H^+ \end{array} \qquad (2)$$

A value of approximately 6.4 for pK_H could be estimated from the experiments with different Cl^- concentrations, and the values of K_A and K_B calculated for $pK_H = 6.4$ are given in Table I. In the experiments with F^-, Cl^-, and Br^- no significant residual inhibition at alkaline pH was observed so that $K_B \gg (I)$. The spectral shift parallels the activity in these cases and can be described with the equation

$$\Delta\epsilon_{640} = (\Delta\epsilon_{640})_{max} \frac{1}{1 + \frac{(H^+)}{K_H}\left[1 + \frac{(I)}{K_A}\right]} \qquad (3)$$

where $\Delta\epsilon_{640}$ is the difference between the observed extinction coefficient and the estimated extinction coefficient at the low-pH extreme. The midpoint of the

TABLE I: Inhibitor Constants, K_A and K_B, obtained from Activity and Spectral Data.[a]

Inhibitor (M)	Spectrum K_A (mM)	Act. (mM) K_A	Act. (mM) K_B
F^-	25	25	...
Cl^- (0.025)	20	17	...
(0.100)	19	16	...
(0.500)	21	26	...
Br^-	8	10	...
I^-	0.9	1.0	55
NO_3^-	0.3	0.4	25
NCO^-[b]	0.007	0.009	0.4

[a] K_H of eq 1 and 3 in the section on Results is assumed to be 4×10^{-7} M. [b] At the concentrations used, the formation of carbamylimidazole can be neglected (*cf.* Stark, 1965). The values of K_A are calculated neglecting the possible inhibition by the 0.1 M Cl^- present in these measurements.

spectral change would then appear at pH = pK_{app} = $pK_H + \log(1 + (I)/K_A)$, which coincides with the pH of half-maximal activity when $K_B \gg (I)$. The enzyme species, E, of eq 2 would consequently seem to have the absorption spectrum characterized by the maxima at 618 and 640 mμ, whereas the spectrum of EHI is different for different inhibitors as seen in Figure 1. For the ions I^-, NO_3^-, and NCO^- the inhibition at alkaline pH values indicates the formation of EI (eq 2), whereas the spectral change still follows eq 3 (Figure 3) as if no EI were formed. The reason for this apparent discrepancy is not yet understood.

Effect of Anion Concentration. (a) In Figure 4A the effects of nitrate concentration on the activity and ϵ_{640}, respectively, are presented according to Johnson *et al.* (1942). The slopes of the straight lines are very close to 1, indicative of 1:1 stoichiometry. (b) Kinetic experiments were performed in mixtures of NO_3^- and Cl^- at a pH (6.75) where both ions inhibit. The results shown in Table II indicate that both ions compete for one binding site. (c) A spectrophotometric titration of the Co(II)–enzyme with cyanate was performed at pH 6.1 and an enzyme concentration (3.75×10^{-4} M) well above the apparent inhibitor constant at this pH (1.2×10^{-5} M, using $K_A = 8 \times 10^{-6}$ M and $pK_H = 6.4$). Under these conditions, the binding of cyanate to sites other than the one linked to the spectral change should be detected. The results presented in Figure 4B show that cyanate binds strongly to one site only. Both inhibition and spectral change thus seem to be due to the binding of one anion per enzyme molecule.

Effects of Substrates on the Absorption Spectrum. (a) HCO_3^- (0.1 M) was added to an unbuffered solution of the Co(II)–enzyme, and the pH was varied by addition of gaseous CO_2. No unique enzyme–HCO_3^- spectrum

TABLE II: Inhibition of Co(II)–Carbonic Anhydrase in Mixtures of Cl^- and NO_3^-, pH 6.75, 25°.[a]

Soln (mM)	$k_2'/K_m \times 10^{-6}$ M$^{-1} \times$ sec^{-1} Calcd: Independent Sites	Calcd: Competition	Found
Cl^- (100)	16.8	16.8	18.6
NO_3^- (100)	0.60	0.60	0.65
Cl^- (95) + NO_3^- (5)	3.5	7.2	8.3
Cl^- (90) + NO_3^- (10)	2.0	4.6	4.3
Cl^- (80) + NO_3^- (20)	1.2	2.6	2.5

[a] Enzyme concentration, 7.0×10^{-8} to 7.0×10^{-7} M. The expected activities for independent sites and for identical sites were calculated according to Fridovich (1963), using the values of K_H, K_A, and K_B given in Table I, and $k_2/K_s = 70 \times 10^6$ M$^{-1} \times$ sec^{-1}.

was found, but the midpoint of the spectral change appeared at pH 7.7. This indicates a rather strong binding of HCO_3^- to EH and might be due to the inhibitory interaction of HCO_3^- with the anion binding site as observed by Kernohan (1964) in his kinetic studies on the reverse reaction. (b) The spectrum of unbuffered Co(II)–enzyme was measured at a high concentration of CO_2 (25 mM). The final pH was 5.3, so the HCO_3^- concentration was approximately 4 mM. Under these conditions the CO_2 dissociation constant can be estimated to be less than 10 mM (*cf.* Figure 2) so that most of the enzyme can be assumed to exist as the inactive EH–CO_2 complex. The observed spectrum was identical within the limits of error with that obtained at low pH in the experiments with HCO_3^-, SO_4^{2-}, or F^- (see spectrum 3 in Figure 1B).

Discussion

The kinetic scheme arrived at here for the Co(II)–enzyme is very similar to that reported by Kernohan (1964, 1965) for untreated bovine carbonic anhydrase, so the exchange of metal ion apparently does not change the catalytic mechanism. In addition to differences in the magnitudes of kinetic parameters and the values of K_A and K_B, the major differences between cobalt and zinc carbonic anhydrases are found in the effects of pH and anions on substrate binding. Neither pH nor anions appear to affect CO_2 binding in the Zn(II)–enzyme (Kernohan, 1965). The variations of K_m for the Co(II)–enzyme (Figure 2) are compatible with E, EI, and EH having about the same substrate dissociation constants, but EHI seems to bind CO_2 considerably more weakly.

The relative activity of the Co(II)–enzyme, previously reported as approximately 45% of the activity of the Zn(II)–enzyme (Lindskog and Malmström, 1962; Lindskog and Nyman, 1964), depends, in fact, on the conditions of measurement, particularly the CO_2 concentration. The magnitude of the pH-independent parameters k_2 and K_s for the Co(II)–enzyme, approximately 1.7×10^5 sec^{-1} and 2.4 mM, respectively, are both approximately five times smaller than the corresponding values reported by Kernohan (1965) for the Zn(II)–enzyme. Consequently, at very low CO_2 concentrations, the Co(II)–enzyme is just as active as the Zn(II)–enzyme (25°). The same relation seems to occur for the two major forms of human carbonic anhydrase. The values of V_M and K_m reported by Gibbons and Edsall (1964) for the Co(II) derivatives of human carbonic anhydrases B and C in phosphate buffers, pH 7.05, 25°, are smaller than the corresponding values for the Zn(II)–enzymes, but the ratios V_M/K_m are essentially the same with both metal ions.

The effects of anions on the visible absorption spectrum of Co(II)–carbonic anhydrase suggest that these ions are bound to the metal ion or to a site very close to it. Most of the ions studied here, however, form only weak complexes with metal ions such as Zn^{2+} and Co^{2+} (Sillén and Martell, 1964). In particular, ClO_4^- is usually considered not to form complexes, but preliminary experiments with this ion indicate an inhibitory power comparable to I^- or NO_3^-. Likewise, the sulfonamide, acetazolamide, a potent carbonic anhydrase inhibitor, is not known to form stable metal complexes, but it affects the visible absorption spectrum of the Co(II)–enzyme, and the metal ion is required for its binding to the enzyme (Lindskog, 1963). Thus, although the metal might be part of the anion binding site, other groupings of the protein structure are probably involved. Strong interactions with anions are known for a number of proteins, for example, serum albumin (Scatchard and Yap, 1964) and ribonuclease (Saroff and Caroll, 1962). A specific interaction of anions with the active site of acetoacetate decarboxylase from *Clostridium acetobutyricum* has been very thoroughly studied by Fridovich (1963). This enzyme is not known to contain any metal ion (Utter, 1961), but the pH dependence and the absolute as well as the relative strengths of anion binding are very similar to these properties of Co(II)–carbonic anhydrase.

Acknowledgments

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Glutamate Biosynthesis in *Acetobacter suboxydans*. VI. Formation from Acetate plus Pyruvate*

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ABSTRACT: *Acetobacter suboxydans* has been reported to lack a demonstrable Krebs tricarboxylic acid, the conventional route for the biosynthesis of vital cell components such as glutamate. In a study of possible precursors of glutamate in this organism, it was found that acetate plus pyruvate can serve as a source of glutamate.

The label in the C_1 carbon of glutamate, however, is not in accord with the formation of this amino acid *via* the Krebs cycle. As a possible route to glutamic acid formation from acetate plus pyruvate, the reverse of Barker's scheme for the fermentation of glutamate in *Clostridium tetanomorphum* has been assessed. The enzymatic formation of citramalate, mesaconate, and β-methylaspartate from acetate plus pyruvate in *A. suboxydans* point to the possibility of participation of this pathway in the biosynthesis of glutamate in this organism.

Despite the fact that *Acetobacter suboxydans* is an obligate aerobe, it has been reported by several investigators that no evidence for activity of either the Krebs tricarboxylic acid cycle or for the complete scheme of glycolysis can be obtained by standard manometric, chemical, and radioactive tracer experiments, to account for the complete terminal dissimilation of glucose (Fewster, 1958; Cheldelin, 1961; King and Cheldelin, 1952, 1954; Hauge *et al.*, 1955; Kitos *et al.*, 1957; Rao, 1957). The phosphorylative scheme of carbohydrate dissimilation known as the pentose cycle accounts for the complete dissimilation of glucose or glycerol in *A. suboxydans* (Kitos *et al.*, 1958; Cheldelin, 1961). Except for the inability to supply immediate precursors of amino acids, the pentose cycle, which is the major carbohydrate dissimilation pathway in *A. suboxydans*, plays a role analogous to the tricarboxylic acid cycle in many other tissues, namely to satisfy the energy requirements.

On the other hand the fact that *A. suboxydans* has considerable and varied biosynthetic capacities is illustrated by the simple composition of the chemically defined growth medium (Stokes and Larsen, 1945; Cheldelin, 1961). The organism must therefore possess an appropriate mosaic of enzymes, and alternate routes were suspected to have functional existence, to account for the biosynthesis of numerous vital cell components (such as amino acids) which would otherwise arise through the Krebs cycle from the carbon skeletons

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