Biophysical Chemistry 26(1987) 217–224
Elsevier

BPC 01140

Kinetics and mechanism of anionic ligand binding to carbonic anhydrase

Bala S. Prabhananda *, Edgar Rittger and Ernst Grell

Max-Planck-Institute for Biophysical Chemistry, Göttingen, and Max-Planck-Institute for Biophysics, Frankfurt, F.R.G.

Accepted 27 February 1987

Anion binding; Carbonic anhydrase; Temperature-jump kinetics

The kinetics of complex formation between Co(II)-carbonic anhydrase B and the anions cyanate, thiocyanate and cyanide has been studied at different pH values employing temperature-jump relaxation spectrometry. Formation of the 1:1 complex occurs via binding of the deprotonated state of the anion to an acidic state of the enzyme. The determined formation rate constants range from 10^8 to 3×10^9 M^{-1} s^{-1} and are two to three orders of magnitude higher than the value estimated for a ligand coordination to the central Co^{2+}, based on a solvate substitution mechanism. These kinetic results strongly indicate that the deprotonated anion binds to an unoccupied coordination position of the protein-bound heavy metal ion in the form of an addition reaction. Upon binding of the anion, the coordination number of the Co^{2+} in the acidic state of the enzyme is increased from four to five. In the case of cyanide, a 2:1 anion complex is also formed. The formation rate constant is 5×10^5 M^{-1} s^{-1} which provides good evidence that this binding process is controlled by a solvate substitution mechanism.

1. Introduction

Carbonic anhydrase (EC 4.2.1.1), originating from mammalian erythrocytes, is a small metalloenzyme which exists in the form of several isoenzymes. It catalyzes the hydration of CO_2, respectively the dehydration of bicarbonate. The native form of the enzyme contains a strongly coordinated Zn^{2+}, which is part of the active-site region. Considerable attention has been devoted to the specificity and function of the coordinated heavy metal ion [1]. Removal of Zn^{2+} is accompanied by the complete loss of enzymatic activity. Only Zn^{2+} and Co^{2+} are capable of reactivating the apoenzyme with full retention of enzymatic activity.

The currently existing X-ray structure analysis of the human enzyme [2] does not enable one to make an unequivocal determination of the coordination geometry and of the coordination number of the bound heavy metal ion, which is coordinated to three histidine side chains of the protein. Thus, at least one coordination position is likely to be occupied by a water molecule. The analysis of X-ray absorption spectra obtained for an unspecified isoenzyme mixture of the bovine Co(II)-substituted protein [3] suggests a coordination number of four for its alkaline state and its complexes with many strong anionic inhibitors and five for its acidic state and some complexes with weak ionic ligands.

Carbonic anhydrase exhibits outstanding dynamic properties and represents the most powerful of all known acid-base catalysts. Its most active isoenzymes reach turnover numbers up to 10^6 s^{-1}, indicating that 10^6 substrate molecules are converted into product per unit time. Therefore, investigation of the kinetic properties and mechanistic aspects is of great interest. With regard to the

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

Correspondence address: E. Grell, Max-Planck-Institute for Biophysics, Heinrich-Hoffmann-Str. 7, D-6000 Frankfurt 71, F.R.G.

* Present address: Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India.

reaction mechanism, the protonation of the active site is assumed to be rate-limiting [4]. Proton transfer is expected to occur from buffer molecules to the active-site region via a shuttle residue at the protein surface. If the turnover number is 10^6 s^{-1}, we can conclude that the rate constants of all other first-order processes such as substrate or product dissociation must be at least as high. Let us consider the binding and dissociation of CO_2 and bicarbonate, respectively. In the case of the uncharged CO_2, which is not assumed to be directly coordinated to the central metal ion, very fast binding (even diffusion-controlled) as well as fast dissociation is likely to occur. Since no direct interaction with the heavy metal ion is known, no differences between the properties of the Zn^{2+}- and Co^{2+}-substituted protein are expected. This situation, however, is different when we consider the binding and dissociation of the anionic bicarbonate, which is assumed to coordinate to the protein-bound heavy metal ion.

According to the Eigen-Wilkins substitution mechanism for complex formation between metal ions and ligands, the final inner sphere coordination occurs via the formation of an outer sphere complex (dissociation constant K_D). If the inner sphere solvate substitution rate constant is k_i, the overall complex formation rate constant is equal to $K_D^{-1}k_i$. In the case of an enzymatic reaction, we have to introduce in addition a probability factor P_0. This factor accounts for the fact that only the collision between ligand and a fraction of the total protein surface can lead to the outer sphere complex formation. Based on this simple model, the overall formation rate constant is $P_0K_D^{-1}k_i$. If we now assume P_0 to be 0.1, K_D to be 0.04 M and k_i to be 4×10^7 and 3×10^5 s^{-1} for solvated Zn^{2+} and Co^{2+}, respectively, according to the metal ion substitution rate constants given in ref. 5, we can calculate an estimated value of the overall formation rate constant for the Zn(II)-protein of 10^8 M^{-1} s^{-1} and 7.5×10^5 M^{-1} s^{-1} for the Co(II)-protein. Based on a dissociation constant for bicarbonate binding to both metalloproteins of 10^{-2} M, we can calculate both bicarbonate dissociation rate constants employing the estimated values for the overall formation rate constant. A value of 10^6 s^{-1} thus results for bicarbonate dissociation from the Zn(II)-protein and of only 7.5×10^3 s^{-1} for the Co(II)-protein. Similar conclusions concerning differences in the dynamic parameters of both proteins have to be expected for small anions other than bicarbonate. This substitution mechanism for anionic ligand binding predicts formation rate constants which are independent of the nature of equally charged ligands for a given metal ion, but different formation rate constants for different metal ions. Thus, the values of these rate constants should depend on the nature of the cation [5]. What can we now conclude in the case of carbonic anhydrase, where the rate-limiting reaction process was assumed to be a proton transfer with a rate constant of approx. 10^6 s^{-1} [4]?

A bicarbonate dissociation rate constant of about 10^6 s^{-1} in the case of the Zn(II)-enzyme would be consistent with the mechanistic aspects indicated above. However, in the case of the more than 100-times slower bicarbonate dissociation from the Co(II)-enzyme, a much faster process with a rate constant of 10^6 s^{-1} of course could no longer act as the rate-determining step in a first-order reaction. Since bicarbonate dissociation would be rate-limiting here the rate of the total catalytic cycle would have to be reduced drastically, as compared to the Zn(II)-enzyme. This simple estimation leads to the main conclusion that Co(II)-carbonic anhydrase would have to be much less catalytically active than Zn(II)-carbonic anhydrase. However, this prediction is not at all consistent with the experimental observation, since both metal ion derivatives exhibit the same enzymatic activity [1].

In order to investigate and elucidate the mechanistic aspects of anion binding to carbonic anhydrase, a kinetic study was carried out employing temperature-jump relaxation spectrometery [6]. Since the Co(II)-substituted protein has spectral properties (e.g., its visible adsorption spectrum) which make it more amenable to spectroscopic studies than the native Zn(II)-enzyme, the experimental studies were performed on a bovine Co(II)-carbonic anhydrase (isoenzyme B). Instead of the weakly bound bicarbonate, stronger coordinating anions such as cyanate, thiocyanate and cyanide were used.

2. Experimental

Bovine carbonic anhydrase was obtained from Miles and Sigma. The B isoenzyme (identical with carbonic anhydrase II) was purified on DEAE-cellulose DE 32 (Whatman) similarly to the method in ref. 7. Zn^{2+} was replaced by Co^{2+} according to ref. 8. After formation of the Co(II)-enzyme, the sample was dialyzed for 3 days against four changes of comparatively large volumes of double-distilled water. The protein concentration was measured photometrically on the basis of an extinction coefficient of 57000 $M^{-1}cm^{-1}$ at 280 nm. The rate constant of hydrolysis of the artificial substrate 4-nitrophenyl acetate (0.05 M Tris-H_2SO_4, pH 8.0, at 25°C; ionic strength 0.1 by Na_2SO_4; 0.4 mM substrate and about 10^{-6} M enzyme) was around 1000 $M^{-1}\,s^{-1}$, which corresponds to 100% activity.

Spectrophotometric titrations at 25°C were performed on a Cary 14, Cary 118 and HP 8450 spectrophotometer, equipped with special cuvette holders for efficient temperature control. The errors of the pK values are estimated to be about ±0.25. The determined equilibrium constants represent apparent values, valid for the particular media used in this study.

Temperature-jump relaxation studies were carried out at 22°C with a spectrometer built by Rabl [9]. The temperature jump induced by Joule heating was about 3°C. The errors of the determined rate constants are estimated to be about ±40%.

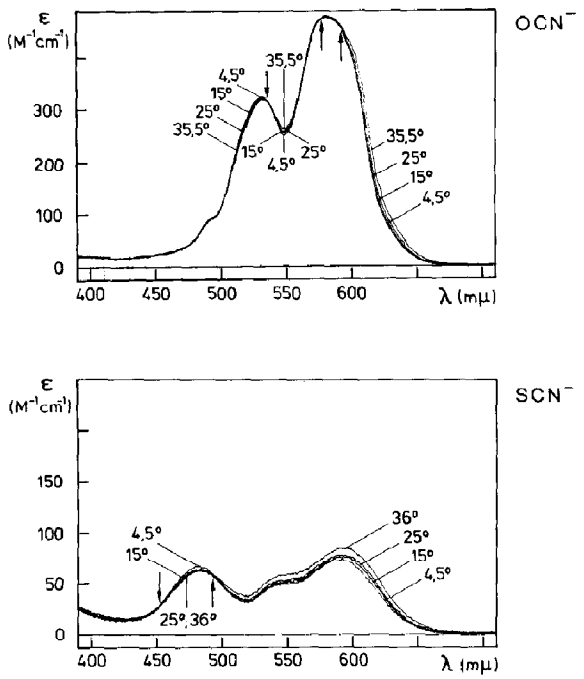
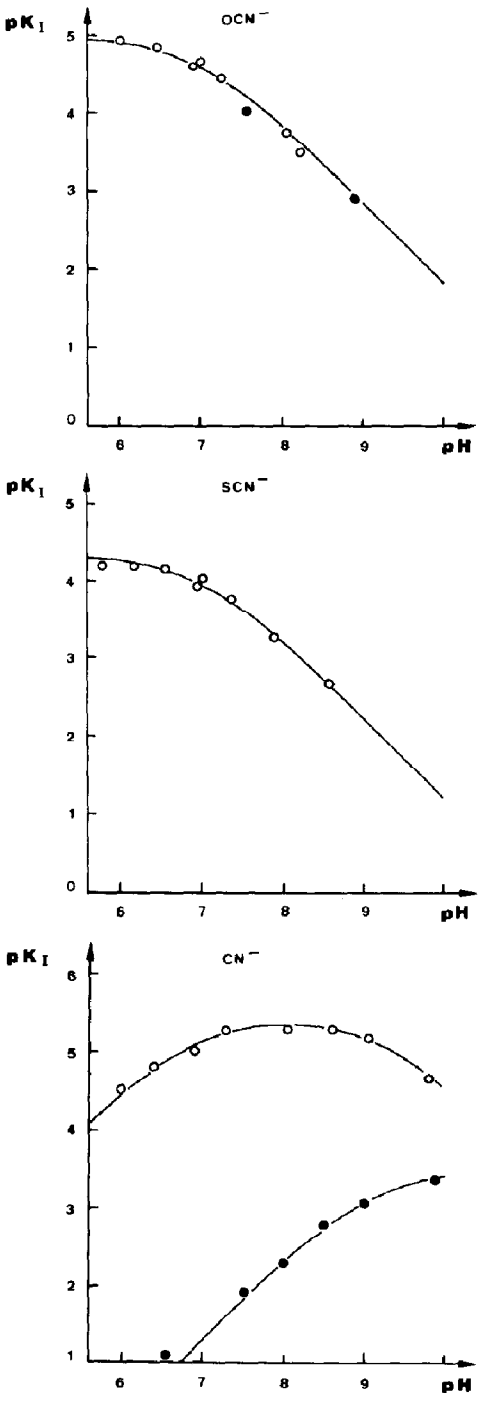


Fig. 1. Temperature dependence of visible absorption spectrum of bovine Co(II)-carbonic anhydrase B in the presence of 0.05 M cyanate and 0.085 M thiocyanate at pH 6.75.

3. Results

3.1. Binding studies

Before carrying out relaxation measurements, it is necessary to determine the equilibrium dissociation constants K_I of the investigated complexes in the medium used for the kinetic studies. Since the temperature-jump instrument used here is equipped for absorption detection, it is furthermore important to measure the absorption spectra of the complexes between bovine Co(II)-carbonic anhydrase B and the anions. Fig. 1 shows the visible absorption spectra (attributed to d-d transitions of Co^{2+}) of the 1:1 cyanate and thiocyanate complexes. The spectra of these complexes exhibit no significant temperature or pH dependence. This suggests that both complexes exist in the form of a rather uniform state. The absorption spectra of the 1:1 and 2:1 cyanide complexes are given in ref. 10. The spectra of all these complexes differ significantly from those of the uncomplexed enzyme. The visible absorption spectrum of free Co(II)-carbonic anhydrase, however, is sensitive to pH which is understood in terms of a pH-dependent, protolytic equilibrium with a pK_{EH} value of 6.8 [1,11]. The overall dissociation constants K_I of the anion complexes can thus be determined either by spectrophotometric titration or from the inhibition of hydrolysis of *p*-nitrophenyl acetate in the presence of bovine Co(II)-carbonic anhydrase B (fig. 2). K_I is



defined as

$$K_I = \frac{[E^\circ][L^\circ]}{[E-L]} \tag{1}$$

where [E°] and [L°] correspond to the total equilibrium concentrations of enzyme and ligand; [E − L] represents that of the complex.

The observed pH dependence of pK_I can be explained by the following reaction scheme

$$\begin{array}{ccccc} H^+ + E & + & LH & & (2) \\ \uparrow\downarrow K_{EH} & & \uparrow\downarrow K_{LH} & & \\ EH & + & L^- + H^+ & \overset{K}{\rightleftarrows} & EH\text{-}L^- + H^+ \end{array}$$

where K_{LH} is the protolytic dissociation constant of the anionic ligand, and K characterizes the complex formation equilibrium between the acidic state of the enzyme EH and the deprotonated state of the ligand L^-. For the pH-dependent dissociation constant K_I we obtain:

$$K_I = K\left(1 + \frac{K_{EH}}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_{LH}}\right) \tag{3}$$

The calculated values of the dissociation constants K for 1 : 1 complex formation with cyanate, thiocynate and cyanide are given in table 1. For pK_{EH} a value of 6.8 was taken and the K_{LH} values used are specified in table 1. The data are consistent with results of earlier studies performed under similar conditions [1,12].

The pH dependence of the dissociation constant characterizing the 2 : 1 cyanide complex was determined by employing spectrophotometric titrations (fig. 2). This additional binding process is only found in the presence of cyanide, which exhibits the strongest ligand field of all the investigated anions. Under conditions where no uncomplexed enzyme exists, the following reaction scheme is postulated

$$\begin{array}{ccc} & LH & \\ & K_{LH}\uparrow\downarrow & \\ EH\text{-}L^- + L^- + H^+ & \overset{K}{\rightleftarrows} & EH\text{-}(L^-)_2 + H^+ \end{array} \tag{4}$$

Fig. 2. pH dependence of overall dissociation constants K_I of different anion complexes at 25 ° C. Data from (○) inhibition studies, (●) spectrophotometric titrations. (—) Calculated according to eqs. 3 and 5 with $pK_{EH} = 6.8$ and the parameters given in table 1.

Table 1

Complex formation between anionic ligands and Co(II)-carbonic anhydrase B: thermodynamic and kinetic parameters

K_{LH}, protolytic dissociation constant; K, complex dissociation constant for binding of deprotonated anion to acidic state of enzyme; k_{12}, formation rate constant for 1:1 complex between deprotonated anion and acidic state of enzyme; k_{21}, corresponding dissociation rate constant; k_{23}, formation rate constant for 2:1 complex involving deprotonated anion; k_{32}, corresponding dissociation rate constant.

Ligand LH	pK_{LH}	pK	k_{12} ($M^{-1}\,s^{-1}$)	k_{21} (s^{-1})	k_{23} ($M^{-1}\,s^{-1}$)	k_{32} (s^{-1})
Cyanate (OCN^-)	3.9	4.9	10^8	10^4		
Thiocyanate (SCN^-)	0.9	4.3	2×10^9	7×10^3		
Cyanide (CN^-)	9.3	7.7 (1:1 complex)	3×10^9	5×10^3		
		3.5 (2:1 complex)			5×10^5	10^3

where

$$K_I = K\left(1 + \frac{[H^+]}{K_{LH}}\right) \tag{5}$$

The experimentally determined K value for 2:1 cyanide complex formation is given in table 1.

3.2. Kinetic studies

The kinetic studies were performed by temperature-jump relaxation spectrometry. Since the Co(II)-enzyme alone in the absence of coordinating anions already exhibits relaxation processes (relaxation times $\tau_{1,2}$) in the millisecond time range [13], studies in the presence of anions could only by performed under limited conditions. Fig. 3 shows a typical relaxation process observed in the presence of cyanate. The corresponding relaxation time τ_3, which is shorter than $\tau_{1,2}$, depends on enzyme concentration and pH. Fig. 4 illustrates the linear dependence of τ_3^{-1} as a function of the sum of the total equilibrium concentrations of enzyme and ligand (calculated from K_I, see fig. 2) according to the kinetic reaction scheme

$$E^\circ + L^\circ \underset{k_{21}}{\overset{k_{12}^\circ}{\rightleftarrows}} E - L \tag{6}$$

with

$$\tau_3^{-1} = k_{12}^\circ([E^\circ] + [L^\circ]) + k_{21}$$

Temperature-jump measurements related to 1:1 complex formation in the presence of cyanate were made at pH 6.0, 6.5, 8.2 and 9.0; with thiocyanate at pH 6.0, 7.05, 7.65 and 8.7; and with cyanide at pH 7.4, 8.0 and 10.2. From the data it follows (see fig. 4 in the case of cyanate) that the dissociation rate constant k_{21} does not depend on pH. Thus, the experimentally observed pH dependence (fig. 4) is attributed to the overall formation rate constant k_{12}°, according to:

$$(k_{12}^\circ)^{-1} = (k_{12})^{-1}\left(1 + \frac{K_{EH}}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_{LH}}\right) \tag{8}$$

where k_{12} represents the pH-independent forma-

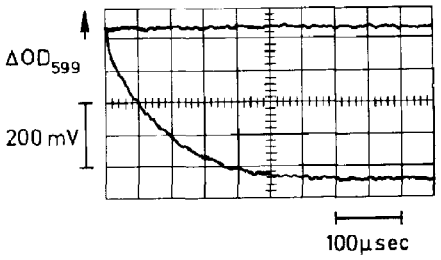


Fig. 3. Temperature-jump relaxation process measured at 22° C and pH 9.05. The sum of the free concentrations of Co(II)-carbonic anhydrase B and cyanate was 6.7 mM. Recording was performed at 599 nm.

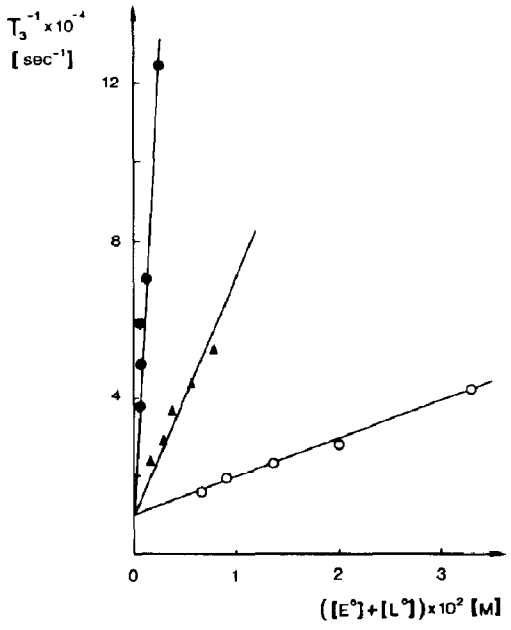


Fig. 4. Relaxation kinetics of 1 : 1 complex formation between cyanate and Co(II)-carbonic anhydrase B. Plot of reciprocal relaxation time τ_3 vs. the sum of the free concentrations of enzyme and ligand at pH 6.5 (●), 8.2 (▲) and 9.0 (○).

tion rate constant which characterizes the binding of the deprotonated anion L^- to the acidic state of the enzyme EH. From the known pK_{EH} value and the thermodynamic data given in table 1, the k_{12} values of the 1 : 1 complexes have been calculated. For the cyanide anion a rate constant of 3×10^9 M^{-1} s^{-1}, for the thiocyanate anion of 2×10^9 M^{-1} s^{-1}, and for the cyanate anion of 10^8 M^{-1} s^{-1} are obtained. The corresponding dissociation rate constants are also listed in table 1.

For principle reasons, the enzyme-ligand complex EH-L^- can be formed on the basis of two different reaction paths, namely, by recombination of EH and L^-, as hitherto assumed, or by recombination of E^- and LH (not shown in eq. 2). If EH predominantly recombines with L^-, the already given value of 10^8 M^{-1} s^{-1} is valid for the formation of the cyanate complex. However, if E^- were to recombine with the protonated state of anion LH, a value of 10^{11} M^{-1} s^{-1} would result for this reaction path. This estimate seems to be unreasonably high for a diffusion-controlled reaction involving a protein molecule. Thus, the reaction path based on the recombination of EH and L^- (as shown in eq. 2) is much more probable and cannot be excluded.

From the kinetic parameters (table 1) of the 1 : 1 complex formation reaction, the ratio k_{21}/k_{12} can be calculated and compared with the corresponding equilibrium constants K. A fairly large deviation of about one order of magnitude results for the cyanate complex. This deviation raises the question as to whether an additional, more stable state of this complex may exist. Indeed, a further relaxation process τ_4 of small intensity in the 300 μs time range was observed under certain conditions in the presence of cyanate. However, whether this relaxation can be attributed to the postulated, additional stabilization process is not yet clear. Also, in the presence of thiocyanate, an extra relaxation process τ_4 has been found. In contrast to the cyanate complex, the relaxation time of the transition τ_4 of the thiocyanate complex is shorter (approx. 5 μs) than that of the anion-binding step τ_3.

At high cyanide concentrations, an intense relaxation process τ_5 in the 100 μs range has been found in addition to that of the faster 1 : 1 complex formation step τ_3. The reciprocal relaxation time τ_5 depends linearly on the sum of the total free concentrations of the 1 : 1 cyanide complex E – L and of cyanide L°, which is consistent with the following reaction scheme:

$$E - L + L^\circ \underset{k_{32}}{\overset{k_{23}^\circ}{\rightleftarrows}} E - (L)_2 \qquad (9)$$

Temperature-jump experiments were carried out at pH 6.4, 7.3, 8.1, 8.5, 8.9 and 10.2. The value of k_{32} is independent of pH. Thus, the experimentally observed pH dependence is attributed to the overall formation rate constant k_{23}° with

$$(k_{23}^\circ)^{-1} = (k_{23})^{-1}\left(1 + \frac{[H^+]}{K_{LH}}\right) \qquad (10)$$

where k_{23} characterizes the recombination between the 1 : 1 cyanide complex of Co(II)-carbonic anhydrase B and the deprotonated state of the anion. The experimentally observed pH depen-

dence of k_{23}^{o} is consistent with eq. 10 and leads to a k_{23} value of 5×10^5 M^{-1} s^{-1}. The value for k_{32} is listed in table 1.

4. Discussion

From the kinetic results obtained for the binding of cyanate to Co(II)-carbonic anhydrase it is concluded that the deprotonated protolytic state of the anion binds to a protonated state of the enzyme:

$$EH + L^- \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} EH - L^- \qquad (11)$$

The acidic group of the enzyme involved in anion binding may be due to a water molecule coordinated to the central Co^{2+} (see fig. 5). ^{13}C-NMR studies [14] have indicated that CN^- and not HCN is bound to this metalloenzyme. Thus, the reaction scheme shown above is also very likely to be valid for anions other than cyanate.

Based on a solvate substitution mechanism, the rate constants of complex formation between anionic ligands and Co(II)-carbonic anhydrase are expected to be around 7.5×10^5 M^{-1} s^{-1} (see section 1) and to be essentially independent of the nature of the anion. However, the data presented in this study (table 1) clearly show that the experimentally determined formation rate constants k_{12} can reach values as high as 3×10^9 M^{-1} s^{-1}. They are two to three orders of magnitude higher than the estimated rate constant. In addition, the k_{12} values of the three ligands investigated differ by more than one order of magnitude. All this evidence strongly supports the conclusion that no solvate substitution can take place upon binding. As the relevant binding mechanism we have to suggest the coordination of the deprotonated anion to a free coordination position of the Co^{2+} (not preoccupied by a solvate molecule) in the form of an addition reaction (see fig. 5). Since the binding site is surrounded by the protein moiety, this reaction is expected to occur in a local region of comparatively low dielectric constant. This may also partly explain why so many anions exhibit surprisingly high affinities to the central heavy metal ion of carbonic anhydrase.

Since no solvate molecule is substituted by the bound anionic ligand, the coordination number of the Co^{2+} must increase upon binding of the anion to the acidic state of the enzyme, as indicated schematically in fig. 5. We thus assign coordination number four to the acidic state of the enzyme (I in fig. 5) and coordination number five to its anion complexes (II in fig. 5). At least as far as the cyanide complex is concerned, this straightforward interpretation is not consistent with conclusions derived from X-ray absorption studies [3] and visible absorption spectra [15]. As possible explanations, we have to consider that possibly more than one state of the anion complexes may exist which can contribute to the equilibrium properties, as indicated by the observation of additional relaxation times τ_4.

Cyanide can also form a higher 2 : 1 complex, due to the binding of a second deprotonated anion to the preformed 1 : 1 complex. The corresponding formation rate constant is 5×10^5 M^{-1} s^{-1} (table 1). This value is now consistent with that expected

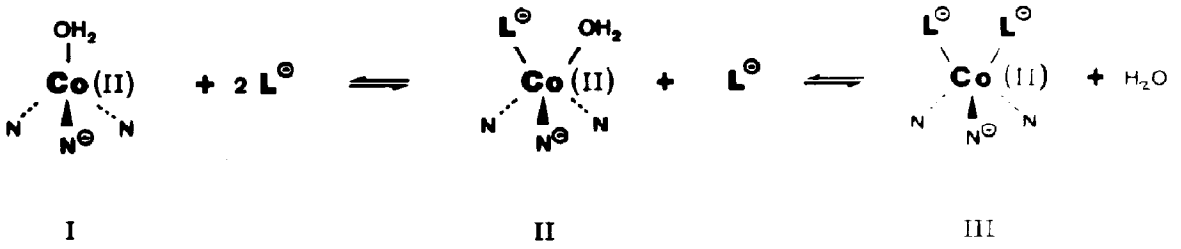


Fig. 5. Schematic illustration of complex formation between anions and Co^{2+} coordinated to three histidine residues of carbonic anhydrase. State III is only significantly populated in the presence of cyanide.

for a solvate substitution process (see section 1). We thus assume that the solvate molecule remaining in the 1:1 complex is substituted by the cyanide ion as shown schematically by state III in fig. 5. Thus, coordination number five of the central Co^{2+} remains upon binding of the second cyanide ion.

For a conclusive discussion of the additional relaxation processes observed which are attributed to first-order rearrangement processes of the 1:1 complexes, further experimental evidence is required.

Acknowledgements

The authors are indebted to T. Claisse, Professors H. Diebler, M. Eigen, T. Kaden, S. Lindskog, I. Pecht, and Dr. H. Ruf for many extensive and fruitful discussions.

References

1 S. Lindskog, in: Structure and bonding, eds. P. Hemmerich, C.K. Jørgensen, N.B. Neilands, R.S. Nyholm, D. Reinen and R.J.P. Williams (Springer, Berlin, 1970) vol. 8, p. 153 and references therein.
2 K.K. Kannan, in: Biophysics and physiology of carbon dioxide, eds. C. Bauer, G. Gros and H. Bartels (Springer, New York, 1980) p. 184.
3 V. Yachandra, L. Powers and T.G. Spiro, J. Am. Chem. Soc. 105 (1983) 6596.
4 D.N. Silverman and S.H. Vincent,: CRC Crit. Rev. Biochem. 14 (1983) 207 and references therein.
5 H. Diebler, M. Eigen, G. Ilgenfritz, G. Maass and R. Winkler, Pure Appl. Chem. 20 (1969) 93.
6 M. Eigen and L. DeMaeyer, in: Techniques of organic chemistry, eds. S.L. Freiss, E.S. Lewis and A. Weissberger (Wiley, New York, 1963) p. 895.
7 S. Lindskog, Biochim. Biophys. Acta 39 (1960) 218.
8 S. Lindskog and B.G. Malmström, J. Biol. Chem. 237 (1962) 1129.
9 C.R. Rabl, in: Technische biochemie, Dechema monographien, ed. H.J. Rehm (Verlag Chemie, Weinheim, 1973) vol. 71, p. 187.
10 E. Grell and R.C. Bray, Biochim. Biophys. Acta 236 (1971) 503.
11 S. Lindskog, J. Biol. Chem. 238 (1963) 945.
12 S. Lindskog, Biochemistry 5 (1966) 2641.
13 E. Grell, B.S. Prabhananda, E. Rittger and H.H. Grünhagen, Inorg. Chim. Acta 79 (1983) 151.
14 J. Feeney, A.S.V. Burgen and E. Grell, Eur. J. Biochem. 34 (1973) 107.
15 I. Bertini, G. Canti, C. Luchinat and A. Scozzafava, J. Am. Chem. Soc. 100 (1978) 4873.