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THE INHIBITION BY ANION BINDING OF REACTIONS OF INORGANIC RADICAL ANIONS WITH BOVINE CARBONIC ANHYDRASE B

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Reactions of the inorganic radical anions, Br_2^- and $(SCN)_2^-$, with bovine carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) have been studied by pulse radiolysis. Reaction is almost completely inhibited by the binding of Br^-, SCN^- and ClO_4^- to an electrophilic site at the active centre of the enzyme. Dissociation constants for anion binding calculated from the reduction in free radical reactivity agree well with inhibition constants for these anions. The anions OCN^- and CN^-, although potent inhibitors of carbonic anhydrase activity, have relatively little effect on the reactivity of radical anions with the enzyme. Reaction of radical anions occurs mainly with tryptophan and tyrosine residues in the hydrophobic core of the enzyme, through a channel at the active site. This channel is closed by the anions in accord with their position in the lyotropic series.

Introduction

The specific binding to the active site of bovine carbonic anhydrase B (carbonate hydro-lase, EC 4.2.1.1) by numerous monovalent anions displaying widely differing potency as inhibitors of enzymic activity is well known [1]. The strongest anion inhibitors such as CN^- and SH^-, are typical 'metal poisons' and it is believed that they interact by co-ordination to the essential Zn^{2+} cofactor of carbonic anhydrase. In contrast, certain of the moderate and weak inhibitors, such as NO_3^- and ClO_4^-, do not normally form stable metal ion complexes. Pocker and Stone [2] have remarked upon the similarities with respect to anion inhibition between carbonic anhydrase and the non-metalloenzyme acetoacetate decarboxylase of *Clostridium acetobutyricum* [3]. The ability of anions to bind to acetoacetate decarboxylase follows the Hofmeister or lyotropic series, which can be related to the formation of stable hydrogen bonds around ion-pairs in solution. The capacity of anions to form stable co-ordination complexes with Zn^{2+} increases in the order $I^- < Br^- < Cl^-$ whilst the inhibitory effect of these anions on carbonic anhydrase is in the reverse order, and follows the lyotropic series. Redpath et al [4] have suggested that binding of SCN^- to carbonic anhydrase affords some degree of protection to internal tryphophan residues from reaction with the radical anion, $(SCN)_2^-$. Another study of the reactions of inorganic radical anions with carbonic anhydrase [5] shows that radical reactivity depends on the presence of Zn^{2+} in the active site of carbonic anhydrase, and on the state of ionization of the activity-linked group with which it is associated, indicating that access to the reactive hydrophobic amino acids is mainly through the region of the active site of this enzyme. For experiments reported here, the reactivity of inorganic radical anions ($(SCN)_2^-$ and Br_2^-) with carbonic anhydrase has been studied by the

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technique of pulse radiolysis (for a review, see [6]) in N_2O-saturated solutions of protein and the respective anion (X^-). Formation of the radical anions ($X_2^{\cdot-}$) occurs within the time of the radiation pulse according to the following sequence of reactions:

$$H_2O \rightsquigarrow {}^{\cdot}OH, {}^{\cdot}H, e_{aq}^-, H_2, H_2O_2, H_3O^+ \quad (1)$$

$$N_2O + e_{aq}^- \rightarrow N_2 + OH^- + {}^{\cdot}OH \quad (2)$$

$${}^{\cdot}OH + X^- \rightarrow X^{\cdot} + OH^- \quad (3)$$

$$X^{\cdot} + X^- \rightleftharpoons X_2^{\cdot-} \quad (4)$$

Methods and Materials

The methods used for purification of carbonic anhydrase, preparation of apo-carbonic anhydrase and experimental aspects of the pulse radiolysis experiments have been described previously [5]. All experiments with apo-carbonic anhydrase were performed with solutions containing 50 μM EDTA to avoid traces of metal ions recombining with the enzyme. At low and high anion concentrations, radical decay was found to fit first- and second-order rate laws as shown in the respective figures. At intermediate anion concentrations, mixed first- and second-order decays were observed, and evaluation of the first-order rate was accomplished by the method of Schmidt [7]. Experimental rate constants have a probable error of 10%. All the experiments were performed in solutions buffered to pH 7.0 with phosphate (1 mM) at 22 ± 2°C.

Results

Reactions of $(SCN)_2^{\cdot-}$ and $Br_2^{\cdot-}$ with carbonic anhydrase

The decay of the 480 nm absorption of the $(SCN)_2^{\cdot-}$ radical in a solution containing 0.1 mM KSCN/2.5 mg/ml carbonic anhydrase at pH 7.0 is shown in the inset of Fig. 1A. A plot of log (absorbance at 480 nm) versus time shows the decay to be pseudo-first-order ($k_1 = 2.0 \cdot 10^4\ s^{-1}$), giving a second-order rate constant $k_2 = 2.5 \cdot 10^8\ M^{-1} \cdot s^{-1}$ for reaction of $(SCN)_2^{\cdot-}$ with carbonic anhydrase. Under similar conditions, except that the SCN^- concentration was increased to 10^{-2} M, the results in Fig. 2A were obtained. The linear plot of $(A_{480})^{-1}$ vs. time at this higher SCN^- concentration shows the $(SCN)_2^{\cdot-}$

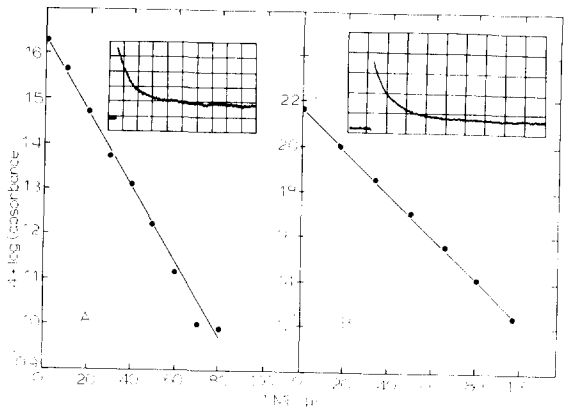

Fig. 1. A: Pseudo-first-order decay of $(SCN)_2^{\cdot-}$ at 480 nm in a solution containing carbonic anhydrase (2.5 mg/ml) and SCN^- (0.1 mM) at pH 7.0. Inset: oscillogram at 480 nm; ordinate-transmission 0.234%/cm; abscissae-time 50 μs/cm. B: Pseudo-first-order decay of $Br_2^{\cdot-}$ at 360 nm in a solution containing carbonic anhydrase (1.5 mg/ml) and Br^- (10mM) at pH 7.0. Inset: oscillogram at 360 nm; ordinate-transmission 1.26%/cm; abscissae-time 50 μs/cm.

decay to be predominantly second-order representing

$$(SCN)_2^{\cdot-} + (SCN)_2^{\cdot-} \rightarrow (SCN)_2 + 2\ SCN^- \quad (5)$$

The slope of this plot gives $k(5) = 2.4 \cdot 10^9\ M^{-1} \cdot s^{-1}$, in good agreement with the results of Baxendale et al. [8] in solution of equivalent ionic strength. The rate

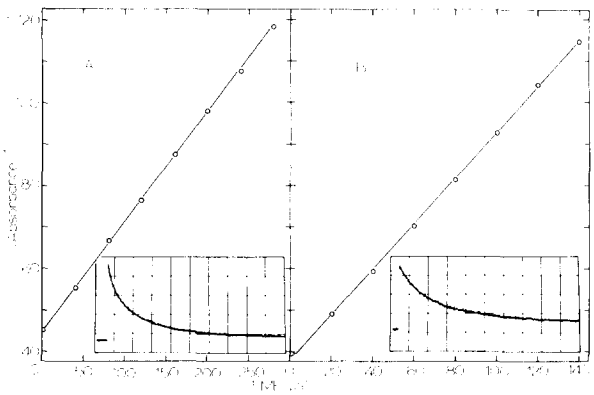

Fig. 2. A: Second-order decay $(SCN)_2^{\cdot-}$ at 480 nm in a solution containing carbonic anhydrase (2.5 mg/ml) and SCN^- (10 mM) at pH 7.0. Inset: oscillogram at 480 nm; ordinate-transmission 1.48%/cm, abscissae-time 200 μs/cm. B: Second-order decay of $Br_2^{\cdot-}$ at 360 nm in a solution containing carbonic anhydrase (1.5 mg/ml) and Br^- (1 M) at pH 7.0. Inset: oscillogram at 360 nm; ordinate-transmission 1.81%/cm; abscissae-time 50 μs/cm.

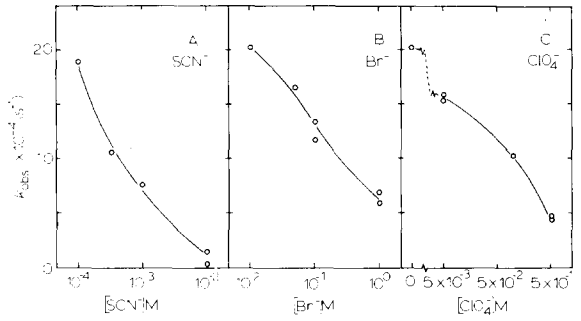

Fig. 3. Anion-induced inhibition of the first-order rate constant (k_{obs}) for reaction of radical anions with carbonic anhydrase. A: Rate of reaction of $(SCN)_2^-$ with carbonic anhydrase (2.5 mg/ml) with increasing $[SCN^-]$. B: Rate of reaction of Br_2^- with carbonic anhydrase (1.5 mg/ml) with increasing $[Br^-]$. C: Rate of reaction of Br_2^- with carbonic anhydrase (1.5 mg/ml) with increasing $[ClO_4^-]$.

constants of the first order component (k_1) of the $(SCN)_2^-$ decay between 0.1 and 10 mM [KSCN] were evaluated by the method of Schmidt [7]. The change in k_1 with $[SCN^-]$ is shown in Fig. 3A.

The inhibition constant (K_i) for SCN^- binding with carbonic anhydrase at pH 7.55 reported by Pocker and Stone [2] is $5.9 \cdot 10^{-4}$ M. Therefore, at 0.1 mM KSCN less than 15% of the carbonic anhydrase would be expected to exist in the SCN^- inhibited state, whereas at 10 mM [KSCN] almost 95% of the carbonic anhydrase molecules are predicted to be binding the SCN^- anion. The decrease in reactivity of $(SCN)_2^-$ towards carbonic anhydrase with increasing $[SCN^-]$ appears to be associated with the binding of the SCN^- anion. The curve in Fig. 3A was analyzed on the assumption that the reactivity of $(SCN)_2^-$ decreases linearly with increasing inhibition of carbonic anhydrase by SCN^-.

The measured rate constant for the first-order decay of the radicalanion (k_{obs}) is the sum of the components for reaction with free enzyme (E_f) and complex (C)

$$k_{obs} = k_e[E_f] + k_c[C] \tag{6}$$

where k_e and k_c are the second-order rate constants for reaction of the radical anion with free enzyme and complex, respectively. Assuming carbonic anhydrase has a single anion binding site, the association constant (K_A) is given by:

$$K_A = \frac{[C]}{[A][E_f]} \tag{7}$$

Since $[C] = [E_o] - [E_f]$, if $[E_o]$ is the total enzyme concentration,

$$k_{obs} = \frac{[E_0](k_e - k_c)}{1 + K_A[A]} + k_c[E_0] \tag{8}$$

when [A] is the concentration of free anion in solution. Generally in these experiments the anion-carbonic anhydrase adduct concentration was very much less than the anion concentration, and [A] can be appriximated to the added anion concentration. For SCN^- the best least-squares fit ($r = 0.986$) of the data in Fig. 3A to Eqn. 8 was found (Fig. 4) when $K_A = 3.6 \cdot 10^3\ M^{-1}$, equivalent to a value of the dissociation constant, $K_D = 2.8 \cdot 10^{-4}$ M, which is reasonably similar to the value of K_i ($5.9 \cdot 10^{-4}$ M) found by Pocker and Stone [2]. This fit to Eqn. 8 also indicates that the rate constant for reaction of $(SCN)_2^-$ with the uninhibited enzyme, k_e, is $2.9 \cdot 10^8\ M^{-1} \cdot s^{-1}$ and that the rate constant for reaction of $(SCN)_2^-$ with the adduct, k_c, is $8.4 \cdot 10^6\ M^{-1} \cdot s^{-1}$, about 3% of k_e. These results are summarized in Table I.

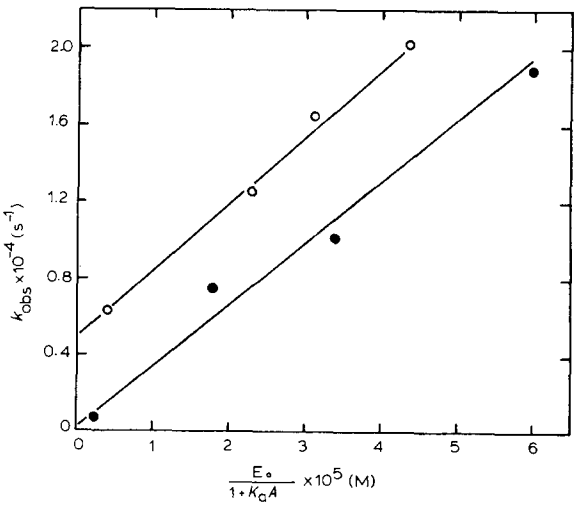

Fig. 4. Plots of Eqn. 8 for: ○ reaction of Br_2^- with carbonic anhydrase inhibited by Br^-, with $K_a = 11.2\ M^{-1}$; ●, reaction of $(SCN)_2^-$ with carbonic anhydrase inhibited by SCN^-, with $K_a = 3.6 \cdot 10^3\ M^{-1}$.

TABLE I

INHIBITION OF REACTIONS OF X_2^- WITH CARBONIC ANHYDRASE BY ANIONS AT pH 7.0

	X_2^- : $(SCN)_2^-$	Br_2^-			
	A^- : SCN^-	Br^-	ClO_4^-	OCN^-	CN^-
k_e [a]	$2.9 \cdot 10^8$	$4.6 \cdot 10^8$	$4.6 \cdot 10^8$	$4.6 \cdot 10^8$	$4.6 \cdot 10^8$
k_c [a]	$8.4 \cdot 10^6$	$1.0 \cdot 10^8$	$8.5 \cdot 10^7$	$3.6 \cdot 10^8$	$4.4 \cdot 10^8$
$(k_c/k_e) \times 100\%$	2.9%	22%	18%	78%	96%
K_D (present work [b])	$2.8 \cdot 10^{-4}$	$8.9 \cdot 10^{-2}$	$1.9 \cdot 10^{-2}$	–	–
K_i [2,9] [b]	$5.9 \cdot 10^{-4}$	$6.6 \cdot 10^{-2}$	$1.6 \cdot 10^{-2}$	$3.9 \cdot 10^{-5}$	$3.2 \cdot 10^{-6}$

[a] Units, $M^{-1} \cdot s^{-1}$.
[b] Units, M.

The decay of the 360 nm absorption of the Br_2^- radical in a solution containing 10 mM KBr and 1.5 mg/ml carbonic anhydrase at pH 7.0 is shown in the inset to Fig. 1B. The reaction is first-order for which $k_1 = 2.0 \cdot 10^4\ s^{-1}$, giving a second-order rate constant $k_2 = 4.2 \cdot 10^8\ M^{-1} \cdot s^{-1}$. On increasing thr KBr concentration to 1 M, the Br_2^- decay becomes predominantly second-order (Fig. 2B). The first order components of the Br_2^- decay at KBr concentrations between 10 mM and 1 M are shown in Fig. 3B. The best least-squares fir ($r = 0.997$) to Eqn. 8 shown in Fig. 4 leads to the values $K_A = 11.2\ M^{-1}$ ($K_D = 8.9 \cdot 10^{-2}$ M); $k_e = 4.6 \cdot 10^8\ M^{-1} \cdot s^{-1}$ and $k_c = 1.0 \cdot 10^8\ M^{-1} \cdot s^{-1}$. With Br_2^-, the Br^--carbonic anhydrase adduct has a reactivity of 22% of that for the uninhibited enzyme. This is shown more clearly when the results are replotted in Fig. 5 as $\%k_{obs}/k_e$ against percentage inhibition. The value of K_D obtained here ($8.9 \cdot 10^{-2}$ M) is in good agreement with the inhibition constant for Br^- ($6.6 \cdot 10^{-2}$ M) at pH 7.55 [2].

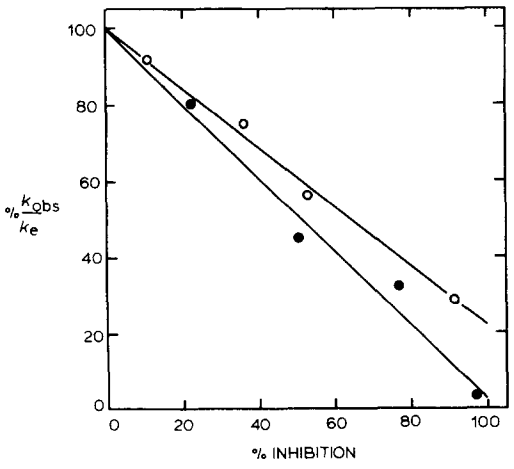

Fig. 5. Relation between percentage rate of radical anion reaction with carbonic anhydrase versus anion-induced inhibition of carbonic anhydrase ○ for Br_2^- reaction inhibited by Br^-; ●, for $(SCN)_2^-$ reaction inhibited by SCN^-.

Inhibition of Br_2^- reactivity with carbonic anhydrase by ClO_4^-

The perchlorate anion is a moderate inhibitor of carbonic anhydrase with $K_i = 1.6 \cdot 10^{-2}$ M [2]. The ability of ClO_4^- to inhibit the reaction of Br_2^- with carbonic anhydrase was assessed in an enzyme solution 1.5 mg/ml) at pH 7.0 containing 10 mM KBr and concentrations of ClO_4^- between 5 mM and 0.5 M. The effect of $[ClO_4^-]$ on the first-order component of the Br_2^- radical anion decay is shown in Fig. 3C. Analysis of this curve is complicated by binding of both Br^- and ClO_4^- to carbonic anhydrase; but if the Br^- component (calculated to be <0.1 mol Br^- bound/mol protein from the value of K_D detemined above) is ignored, Eqn 8 can be used. This simplified treatment gives a value of $K_D = 1.9 \cdot 10^{-2}$ M, in close agreement with the value of $K_i = 1.6 \cdot 10^{-2}$ M for ClO_4^- [2]. The results also indicate that the ClO_4^- inhibited carbonic anhydrase has a reactivity with Br_2^- of about 20% that of the uninhibited enzyme.

Inhibition of Br_2^- reactivity with carbonic anhydrase by other anions

The most potent anion inhibitors of carbonic

anhydrase are HS^- ($+H_2S$) ($K_i = 1.9 \cdot 10^{-6}$ M), CN^- ($K_i = 3.2 \cdot 10^{-6}$ M) and OCN^- ($K_i = 3.9 \cdot 10^{-5}$ M) [9]. Attempts were made to assess the change in reactivity of Br_2^- with carbonic anhydrase caused by the binding of these anions.

Addition of OCN^- (1 mM) to carbonic anhydrase solution (1.5 mg/ml) at pH 7.0 should lead to 96% inhibition of the carbonic anhydrase according to the K_i value quoted above. The reactivity of Br_2^- with the OCN^- adduct of carbonic anhydrase was measured by addition of 50 mM KBr to the above solution. Competition in the binding of carbonic anhydrase by Br^- leads to 0.94 mol OCN^- boind/mol carbonic anhydrase and 0.02 mol Br^- bound/mol carbonic anhydrase. Hence, the effect of Br^- binding is negligible. The average rate constant measured for the Br_2^- first-order decay under these conditions was $1.7 \cdot 10^4$ s^{-1}. This compares with a value of $k_1 = 2.2 \cdot 10^4$ s^{-1} for the reaction of Br_2^- with carbonic anhydrase inhibited to the extent of 2%, estimated from the results in section 3.1. Whilst OCN^- is a powerful inhibitor of carbonic anhydrase activity, the OCN^--carbonic anhydrase complex retains approx. 80% of its reactivity with Br_2^-.

The first-order rate constant for the Br_2^- radical anion decay in a solution of carbonic anhydrase (1.5 mg/ml) containing KCN (0.1 mM) and KBr (10 mM) was found to be $2.1 \cdot 10^4$ s^{-1}. Under these conditions, according the value of K_i for CN^- [9] 97% of the enzyme is inhibited by CN^-, whilst Br^- binding is less than 0.5%. Clearly the carbonic anhydrase retains more than 95% of its reactivity with Br_2^- on formation of the CN^- adduct.

The effects of SH^- binding to carbonic anhydrase on Br_2^- reactivity could not be measured due to a rapid reaction between Br_2^- and free SH^- in solution ($k = 3.4 \pm 1.0\ ^-10^8$ $M^{-1} \cdot s^{-1}$). The rapid oxidation of SH^- by Br_2^- is not surprising since cysteine is also rapidly oxidised by Br_2^- ($k = 1.8 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at pH 6.6 [10]). Unlike bisulphide, the order components used in this work, such as phosphate (1 mM), EDTA (0.05 mM) and KOCN (1 mM), did not affect the second-order decay of Br_2^- in solution at pH 7.0.

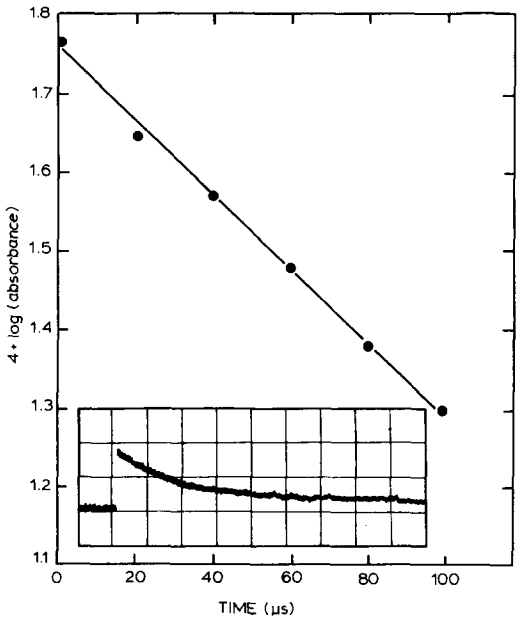

Fig. 6. Pseudo-first-order decay of $(SCN)_2^-$ absorption at 480 nm in a solution containing apo-carbonic anhydrase (2.5 mg/ml), SCN^- (0.1 mM) and EDTA (50 μM) at pH 7.0. Inset: oscillogram at 480 nm, ordinate-transmission 0.78%/cm; abscissae-time 50 μs/cm.

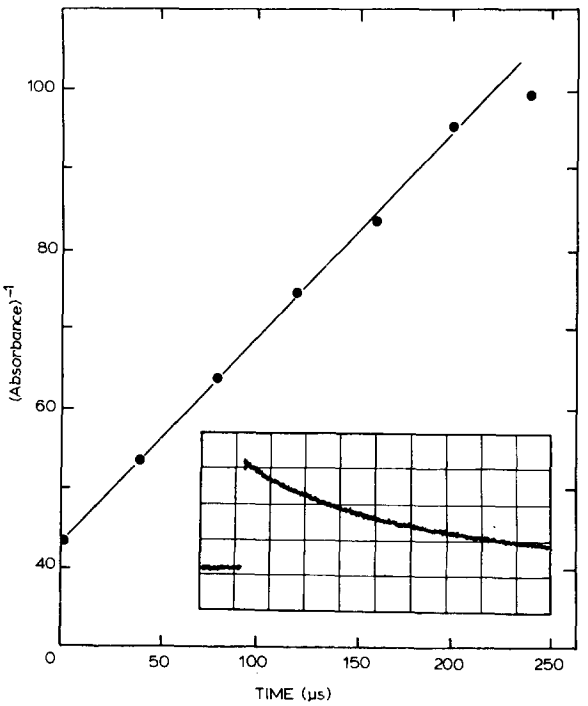

Fig. 7. Second-order decay of $(SCN)_2^-$ absorption at 480 nm in a solution containing apo-carbonic anhydrase (2.5 mg/ml), SCN^- (10 mM) and EDTA (50μM) at pH 7.0. Inset: oscillogram at 480 nm, ordinate-transmission 1.67%/cm; abscissae-time 50 μs/cm.

Reaction of $(SCN)_2^-$ with apo-carbonic anhydrase

The rate constants for reaction of $(SCN)_2^-$ with Zn^{2+}-free apocarbonic anhydrase (2.5 mg/ml) in a solution containing EDTA (50 μM) were measured at SCN^- concentrations of 0.1 and 10 mM. At 0.1 mM, the $(SCN)_2^-$ decay was pseudo-first order with $k_1 = 1.0 \cdot 10^4\ s^{-1}$ (Fig. 6), giving a value of the second-order rate $k_2 = 1.3 \cdot 10^8\ M^{-1} \cdot s^{-1}$. This is slower than the reaction of $(SCN)_2^-$ with the holoenzyme ($k_2 = 2.3 \cdot 10^8\ M^{-1} \cdot s^{-1}$) at the same SCN^- concentration. On increasing the SCN^- concentration to 10 mM the decay of $(SCN)_2^-$ in the solution of apo-carbonic anhydrase was predominantly second-order (Fig. 7) with $k(5) = 1.8 \cdot 10^9\ M^{-1} \cdot s^{-1}$, showing that the inhibition of he $(SCN)_2^-$ reaction by SCN^- to be similar for the apoenzyme as compared with holoenzyme.

Discussion

The correlation between K_I values for anion binding resulting in decreased radical anion reactivity, and the K_i values for enzyme inhibition from the work of Pocker and Stone [2] show that the same binding phenomena are being observed. The way in which the data fit Eqn. 8 to produce these values of K_I suggest that the model, in which anion binding prevents free radical oxidation of amino acid residues, is correct. Another mechanism which results in diminished reactivity of $(SCN)_2^-$ with tryptophan residues of proteins [11,12] through free radical equilibria, cannot be justified in the present case. Such equilibria were previously only observed in the reactions of $(SCN)_2^-$; no evidence for a free radical equilibrium was found for reaction of Br_2^- with tryptophan. In the present study, reactions of both $(SCN)_2^-$ and Br_2^- are found to be inhibited not only by SCN^- and Br^-, but also by ClO_4^- which is not so readily oxidizable [14]. Additionally, the reversible equilibrium between SCN^- and tryptophan (TrpH) in the oxidised state:

$$(SCN)_2^- + TrpH \rightarrow TrpH^+ + 2\ SCN^- \quad (9)$$

$$SCN^- + TrpH^+ \rightleftharpoons TrpH + SCN^\cdot \quad (10)$$

$$SCN^\cdot + SCN^- \rightleftharpoons (SCN)_2^- \quad (11)$$

$$TrpH^+ \rightleftharpoons Trp^\cdot + H^+ \quad (12)$$

was found only to occur with the protonated tryptophan radical ($TrpH^+$, $\lambda_{max} = 575$ nm). The pK of reaction 12 is 4.3 for free tryptophan [11] and 2.3 for one or more tryptophan residues of pepsin [12]. At pH 7.0, the tryptophan radical product from reactions of $(SCN)_2^-$ with carbonic anhydrase has a long wavelength absorption at 520 nm [5] clearly indicating the neutral radical. Hence, such equilibria cannot account for the effects observed with carbonic anhydrase.

The transient spectrum obtained following reaction of $(SCN)_2^-$ with carbonic anhydrase [5] clearly shows oxidation of both tyrosine and tryptophan residuea. Anion binding therefore does not block the reactivity of a single site, but of the whole molecule. The reduction in $(SCN)_2^-$ reactivity with carbonic anhydrase at 10 mM SCN^- to <3% of the original value also demonstrates this point. This reduction in radical anion reactivity by anion binding would be possible if the reactive sites in the enzyme (tryptophan and tyrosine residues) were buried within the protein structure, and only available through a channel which the bound anion is able to block. This channel must be very near to or actually part of the active site, considering the inhibitory effect of these monovalent anions on carbonic anhydrase activity. $(SCN)_2^-$ reacts selectively with tryptophan and tyrosine residues; Br_2^- also reacts with histidine residues [10]. Much spectroscopic and chemical evidence shows that all seven tryptophan residues and 7 of the 8 tyrosine residues form an aromatic cluster in the interior of the molecule. The remaining tyrosine residue is partly buried, titrating with a pK_a of 10.8. Of the 11 histidine residues in carbonic anhydrase, NMR studies show that between 5 and 8 titrate normally, the highest pK value being 6.4 [13]. The reduction in $(SCN)_2^-$ reactivity to <3% of the original value by SCN^- binding is therefore possible according to the proposed model. Binding of Br^- or ClO_4^- only reduces the reactivity of Br_2^- to ≃20% of the original value. This can now be seen to be due at least partly to the reaction of Br_2^- with the histidine residues which are exposed to the aqueous solvent.

The order of effectiveness of the monovalent anions studied in inhibiting radical anion reactivity is:

$$SCN^- > ClO_4^- > Br^- >> OCN^- \geqslant CN^-$$

This is not the same ordering of the anions according to their inhibitory effects on carbonic anhydrase.

However, these anions may be divided into two groups. Firstly, OCN^- and CN^- (and SH^-) may be considered as typical 'metal poisons' and bind strongly with the Zn^{2+} in the active site to form inner-sphere complexes [1]. The anions SCN^-, ClO_4^- and Br^- do not typically form such metal ion complexes, but have differential water binding properties and are usually ranked in the Hofmeister or lyotropic series. The effectiveness of these anions in causing a decrease in radical anion reactivity corresponds to their position in the lyotropic series. Pocker and Stone [2] have proposed that the anions of the lyotropic series bind to the protonated imidazole ring of a histidine residue at the active site, since their binding competes with binding of the more potent anion inhibitors to Zn^{2+}. The relative inhibitory effects of anions of the lyotropic series on radical anion reactivity found here corresponds to the inhibitory actions of the same anions on acetoacetate decarboxylase, a non-metalloenzyme [3]. The ordering of water molecules on the binding of anions to acetoacetate decarboxylase increases in the order $Br^- < ClO_4^- < SCN^-$, as does the ability of these anions to hinder radical anion attack upon carbonic anhydrase.

The interpretation concerning the binding site of anions of the lyotropic series at a site independent of Zn^{2+} is strengthened by the observation that the reactivity of $(SCN)_2^-$ with Zn^{2+}-free apo-carbonic anhydrase is reduced by SCN^- in a very similar way as with the holoenzyme.

Acknowledgement

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