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FREE RADICAL REACTIONS WITH PROTEINS AND ENZYMES

THE INACTIVATION OF BOVINE CARBONIC ANHYDRASE B

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A comparison of the inactivation of bovine carbonic anhydrase B (carbonate hydro-lyase, EC 4.2.1.1) by $^{\cdot}OH$, $(SCN)_2^{\cdot-}$ and $Br_2^{\cdot-}$ shows that the enzyme contains one or more essential tryptophan residues. Direct oxidation of histidine and tyrosine residues by the radicals is less important in causing inactivation of the enzyme. The effectiveness of all these radicals in inactivating carbonic anhydrase decreases with increasing pH in the region where the activity-linked ionizable group dissociates. Differences between the rates of reaction of $Br_2^{\cdot-}$ and $(SCN)_2^{\cdot-}$ with the holo- and apo-enzyme and between the resulting transient product spectra indicate that access to the reactive tyrosine and tryptophan residues is diminished by the presence of Zn^{2+} in the active site region.

Introduction

It is known that reactive free radical species such as $^{\cdot}OH$ and $O_2^{\cdot-}$ are products of some biochemical reactions [1], in addition to being produced in irradiated aqueous systems. In order to characterize the damage to proteins produced by such species, reactions of radiation-produced free radicals with bovine carbonic anhydrase B (carbonate hydro-lyase, EC 4.2.1.1) have been studied in radiation inactivation measurements and by pulse radiolysis.

After the generation of both oxidizing ($^{\cdot}OH$) and reducing free radicals (e^-_{aq} and $^{\cdot}H$) by γ-irradiation of aqueous solutions:

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

scavengers may be employed to produce secondary radicals, of the type $(X_2)^{\cdot}$; by

$$e^-_{aq} + N_2O \rightarrow N_2 + OH^- + OH^{\cdot} \qquad (2)$$

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

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

These radical anions are less powerful oxidizing species than the initial hydroxyl radical and are consequently more selective in their reactions with organic compounds. The inorganic radical anions ($Br_2^{\cdot-}$ and $(SCN)_2^{\cdot-}$) possess selectively in their reactions with amino acid residues in enzymes [2], and may be used to examine the role of some amino acid residues in the activity of enzymes.

Bovine carbonic anhydrase B is a well characterized enzyme which contains essential Zn^{2+} in the active site [3,4]. Redpath et al. [5] have assessed the role of the metal ion in determining the susceptibility of the enzyme to free radical inactivation. Only in

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reactions involving reducing radicals was a protective effect of Zn^{2+} found.

We have extended this work by examining free radical inactivation of carbonic anhydrase as a function of pH in ranges where ionization of the activity-linked group, and also of the single tyrosine, which occurs within the pH stability range of the enzyme. It was necessary to use a highly purified form of carbonic anhydrase and in the experiments with anionic scavengers, some of which are strong inhibitors of carbonic anhydrase, care was taken to adjust concentrations to ensure that only negligible amounts of anion-binding and consequent inhibition ocurred.

Materials and Methods

Enzyme purification

Commercial bovine carbonic anhydrase (Sigma Type C7500) was found to give several bands on polyacrylamide gel electrophoresis, and was purified by the method of Lindskog [6].

Ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) in 0.01–0.08 M Tris-HCl buffer, pH 8, resolved enzymic activity into two peaks, corresponding to carbonic anhydrases A and B. The B fraction was more abundant and was taken alone and the Tris-HCl buffer removed by gel chromatography on Sephadex G-25 using distilled water as eluant. The enzyme solutions were additionally dialysed against triply-distilled water before radiolysis. Using these procedures the specific activity (see below) of the purified carbonic anhydrase was measured as 2070 units/g compared with 1180 units/g for the Sigma preparation. Carbonic anhydrase concentrations were measured spectrophotometrically, assuming ϵ (280 nm) $5.6 \cdot 10^4 \ l \cdot mol^{-1} \cdot cm^{-1}$ and a molecular weight of 31 000 [6].

Zinc-free carbonic anhydrase was prepared by the method of Lindskog and Malmstrom [7]. A solution of the purified enzyme (approx. 4 g/l) was dialysed against a sodium acetate buffer (0.1 M, pH 5.0) containing *o*-phenanthroline (10^{-2} M). After 2 days residual carbonic anhydrase activity was found to be <2% of the original, but nearly full activity could be restored on addition of zinc sulphate. The resulting apo-carbonic anhydrase solution was dialysed against triply-distilled water to remove acetate and *o*-phenanthroline. In order to avoid recombination of metal ions with apo-carbonic anhydrase, all glassware used in the experiments was rinsed with an EDTA solution and EDTA ($5 \cdot 10^{-5}$ M) was added to the solutions.

Activity measurements

The esterase activity of carbonic anhydrase was measured with *p*-nitrophenyl acetate as substrate. The increase in absorbance at 348 nm of a reaction mixture containing $2 \cdot 10^{-3}$ M *p*-nitrophenyl acetate and 17 mg/l carbonic anhydrase in phosphate buffer, pH 7.6, was measured in a double-beam spectrophotometer at 25°C, with a solution of $2 \cdot 10^{-3}$ M *p*-nitrophenyl acetate as the blank. One unit of carbonic anhydrase activity is defined as the quantity of enzyme catalysing the hydrolysis of 1 μmol *p*-nitrophenyl acetate per min at 25°C.

Reagents

Whenever possible all reagents were of Analar grade. The gases used were of the highest purity commercially available. Water was purified by the usual method of triple distillation from solutions of acidic dichromate and alkaline permanganate.

Irradiations

For the inactivation experiments, solutions were irradiated with ^{60}Co γ-rays in a vessel similar to that described by Howard-Flanders and Alper [31] through which the appropriate gas was bubbled for 20 min prior to, and during, irradition. Dose rates of between 3 and 6.5 Gy/min were employed, and determined using the Fricke dosimeter solution, assuming the G value (number of molecules or atoms formed per 100 eV of adsorbed energy) for Fe^{3+} production to be 15.5. Solutions of carbonic anhydrase ($5 \cdot 10^{-2}$ g/l) were prepared in clean baked glassware, using triply-distilled water as diluent. The pH of the solution was adjusted with phosphate (10^{-3} M) and by addition of NaOH or dilute $HClO_4$ when necessary.

Pulse radiolysis experiments were peformed at the Cancer Research Campaign Gray Laboratory using an electron linear accelerator (1.8 MeV, 0.2 μs pulses), details of which have been published [8]. The methods of solution preparation and handling have been described by Willson [9]. Transient absorptions were recorded on polaroid film and kinetic parameters determined using a Datalab DL 905 transient recorder and a WANG 2200 computer system [10]. The dosimeter was calibrated by pulse irradiation of an aerated KSCN solution (10^{-2} M), assuming the $(SCN)_2^{\cdot -}$ radical produced had $G = 2.8$ and ϵ 7600 l. mol^{-1} · cm^{-1} at 480 nm [11]. When recording transient spectra a dose of approx. 4 Gy/pulse was employed. For kinetic measurements a dose of approx. 2 Gy/pulse was employed. All second-order rate constants for reactions of radical anions with carbonic anhydrase were evaluated from plots of the first-order rate for radical absorption disappearance vs. enzyme concentration. Spectra are presented as plots of radical G value X ϵ vs. wavelength.

Results and Discussion

Primary radicals

Inactivation by primary radicals. To investigate the relative efficiencies of the primary radicals ($^{\cdot}OH$, $^{\cdot}H$ and e^-_{aq}), formed by water radiolysis, in activating carbonic anhydrase, experiments were undertaken in solutions saturated with argon, N_2O or O_2 where the relative free radical yields are modified. The results are shown in Fig. 1.

In all three cases the semilogarithmic survival curves were found to exhibit a shoulder preceding an exponential rate of inactivation. In order to quantify the results, the slope of the exponential region was extrapolated back to 100% activity and the resulting D_{37} of this component allowed a G(inact) value (number of enzyme molecules inactivated per 100 eV energy absorbed) to be calculated. The dose at which the extrapolated line meets 100% activity is designated D_T, the threshold dose. This analysis is more meaningful than simply obtaining an overall D_{37} from which no significant G(inact) value can be calculated. The shape of the curves may be explained by supposing that within the threshold dose region attack on non-essential residues is favoured in competition with attack at less accessible essential residues. However, upon accumulation of damage, reaction with essential residues gradually becomes more favoured. Eventually normal competition between active and inactive enzyme molecules for reaction with the radical species occurs and the usual exponential loss of activity is observed at higher doses. The relative reactivities of essential and non-essential sites is expected to vary with the particular radical being considered. In support of this, the ratio of D_T to G(inact) varies with the saturating gas (Fig. 1) and scavenger present (such as Br^- and SCN^-).

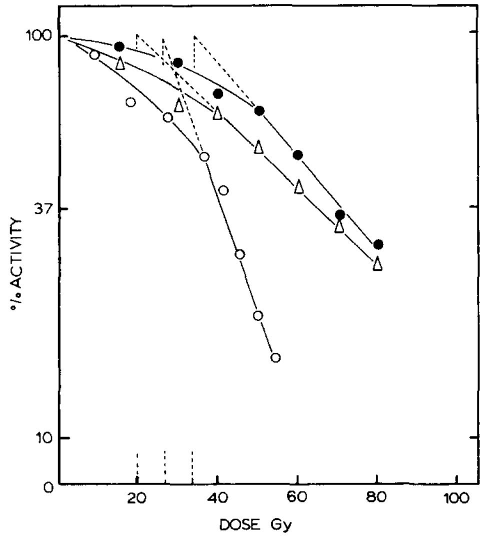


Fig. 1. Inactivation by γ-irradiation of carbonic anhydrase (0.05 mg/ml, in 1 mM phosphate buffer pH 7.0) in solutions saturated with: ○, nitrous oxide; △, oxygen; ●, argon.

Analysis of the results as in Fig. 1 leads to average D_T values of 34,20 and 22 Gy in argon, oxygen and nitrous oxide, respectively. The G(inact) values from the extrapolated semilogarithmic portion of the curve are 0.44, 0.34 and 0.98 in argon, oxygen and nitrous oxide. The G(inact) values show that the effect of N_2O compared with argon is to approx. double the inactivation value due to conversion of e^-_{aq} to $^{\cdot}OH$ and that e^-_{aq} is not an efficient inactivating species.

This assumption is justified by the result that in the presence of O_2 the G(inact) value is only slightly reduced from that in the argon after scavenging both e^-_{aq} and $H^{\cdot}$ by O_2:

$$^{\cdot}H + O_2 \rightarrow HO^{\cdot}_2 \qquad (5)$$

$$\downarrow\uparrow H^+$$

$$e^-_{aq} + O_2 \rightarrow O^{\cdot -}_2 \qquad (6)$$

At pH 7, $O_2^{\cdot -}$ is the predominant radical, a species ineffective in inactivating carbonic anhydrase [5], lysozyme and ribonuclease [12].

The D_T values in oxygen and N_2O are similar and must represent non-inactivating reactions of ˙OH (and possibly $O_2^{\cdot -}$ in the O_2 saturated solution). In argon the D_T value is larger. This might imply some mutual repair involving the oxidizing effect of OH and the reducing effect of e_{aq}^- and H as observed in loss of activity of hemocyanin by Ke and Schubert [13], and also inferred in the back reaction of electrons with photoionised tryptophan residues in lysozyme [14].

These results imply that at pH 7 the major inactivating radical is ˙OH. The difference between the slopes of the exponential regions of the survival curves in O_2 and argon is small ($\Delta G = 0.1$) compared with the G value for inactivation in N_2O saturated solutions (0.98). Hence inactivation by ˙H can be largely ignored in N_2O-saturated solutions.

Since the interpretation of the radical anion results requires studies over the available pH range, the inactivation of carbonic anhydrase by ˙OH in N_2O-saturated solution was studied between pH 5 and 10.7. A similar analysis in terms of D_T and G(inact) values to that described above was used to obtain the results shown in Fig. 2. The sensitivity to inactivation, G(inact), is greatest near pH 7 and falls to a constant value above pH 8. The reduction in sensitivity to ˙OH radicals between pH 7 and 8 corresponds to a pK of 7.5 measured for a species involved in hydrolysis of p-nitrophenyl acetate [15]. It has been suggested that this group in the carbonic anhydrase active site is either a histidine residue ionizing to produce a imidazolate anion co-ordinated to Zn^{2+} in the active site [16] or a Zn^{2+}-bound water molecule dissociating to form a Zn^{2+}-bound hydroxide ion [17]. These results show that the state of ionization of this group effects the reactivity of this site with the ˙OH radical, or affects the accessibility of other nearby groups in the active site to ˙OH radicals.

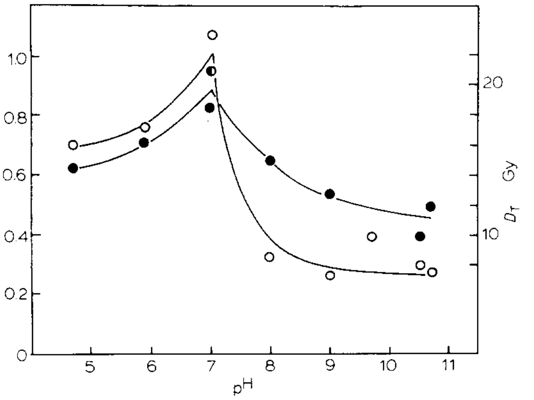


Fig. 2. Inactivation G value (Ginact) (o——o) and threshold dose (D_T) (•——•) as a function of pH for carbonic anhydrase (0.05 mg/ml) irradiated in N_2O-saturated solutions.

The value of D_T also shows a maximum near pH 7 and the ratio of D_T to G(inact) varies over the range of pH studies. According to the model outlined above this would indicate that the relative reactivities of essential and non-essential sites, and the contribution of non-essential sites to D_T, changes according to subtle changes in conformation with pH and to the changing reactivities of sites which ionize over the pH range studied.

Pulse radiolysis studies of the reactions of ˙OH and e_{aq}^-. By competition with SCN^- at 10^{-4} M, the rate constant for reaction of ˙OH with carbonic anhydrase was found to be $6.5 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ at pH 7.0, taking k (˙OH + SCN^-) = $1.1 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ [18]. Higher SCN^- concentrations were not employed due to the possibility of inhibition of free radical reactivity by SCN^- binding to the enzyme [32].

The transient spectrum formed by reaction of ˙OH radicals with carbonic anhydrase at pH 7.0 was measured by pulse radiolysis of an N_2O-saturated carbonic anhydrase (0.62 g/l) solution. The observed spectrum (Fig. 3) is very similar to that from ˙OH attack on trypsin [19] and subtilisin Novo [20]. The spectrum constitutes a mixture of the tryptophan-˙OH radical adduct (λ_{max} 310–320 nm) and a shoulder at approx. 410 nm due to the phenoxy radical derived from tyrosine, although at pH 7 dehydration of the initial dihydroxyphenylalanyl radical ($\lambda_{max} < 300$ nm) is not complete [21,22]. Radicals formed by hydrogen abstraction at the peptide bond units are also expected [23], but as these peptide radicals have absorption maxima below 300 nm with low extinction coefficients, the formation of these radicals cannot be confirmed by the spectra obtained from carbonic anhydrase. The transient spectra obtained by oxidation of carbonic anhydrase by the ˙OH radical is comparatively featureless compared with those from the radical anions, indicative of the general lack of specificity of the ˙OH radical. A

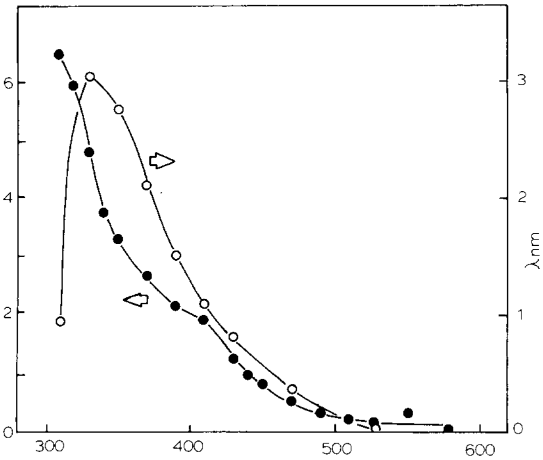


Fig. 3. Transient product spectra from pulse radiolysis of carbonic anhydrase solutions at pH 7.0: ●, N_2O-saturated solutions, [carbonic anhydrase] = 0.62 mg/ml, 10 μs after the pulse; ○, N_2-saturated solutions containing 0.2 M *t*-butanol, [carbonic anhydrase] = 0.48 mg/ml, 30 μs after the pulse.

similar spectrum from the $^{\cdot}OH$ radical reaction with carbonic anhydrase (data not shown) was observed at pH 10.5. In view of the fact that bovine carbonic anhydrase contains only a single partially exposed tyrosine residue with a pK_a of 10.8, it is to be expected that little more reaction at tyrosine residues would be observed at this higher pH. By following the pseudo-first-order decay of e^-_{aq} at 650 nm, the second-order rate constant for reaction of e^-_{aq} with carbonic anhydrase was found to be $1.4 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$. The spectrum of the products formed by reaction of e^-_{aq} and $^{\cdot}H$ with carbonic anhydrase was measured at pH 7.0 in N_2-saturated solutions of carbonic anhydrase (0.48 g/l) containing *t*-butanol (0.2 M) as an $^{\cdot}OH$ radical scavenger (Fig. 3). Since bovine carbonic anhydrase B does not contain disulphide bonds, the intense absorption at 410 nm due to $RSSR^{\cdot}$ observed in many proteins [24] is absent. An absorption maximum at approx. 360 nm, attributable to e^-_{aq} attachment to histidine residues [25] is absent, either because the pH of the solution is above the pK_a of the histidine residues or they are inaccessible from the solvent. The spectrum therefore must represent the attachment of e^-_{aq} to the reactive carbonyl bonds of the polypeptide chain [25].

Inorganic radical anion reactions

Inactivation by inorganic radical anions. In N_2O-saturated solutions containing Br^- or SCN^- the $OH^{\cdot}$ radical is quantitatively converted to $Br_2^{\cdot-}$ or $(SCN)_2^{\cdot-}$ (reactions 3 and 4).

In experiments involving anions, the possibility of the specific binding of such anions to carbonic anhydrase, which results in inhibition of the enzyme, has to be considered: for Br^- $K_i = 6.6 \cdot 10^{-2}$ M and for SCN^- $K_i = 5.9 \cdot 10^{-5}$ M at pH 7.55 and 25°C [26]. In the inactivation studies such low concentrations of Br^- and SCN^- were used that inhibition was minimised. Bromide presented no difficulty: at 5 mM KBr and 1.6 μM carbonic anhydrase there was no detectable loss of activity of native enzyme due to ion binding and calculation shows that >98% of $^{\cdot}OH$ radicals react with Br^-, assuming k ($^{\cdot}OH$ + carbonic anhydrase) = $6.5 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ and $k\,(^{\cdot}OH + Br^-) = 1 \cdot 10^9\ M^{-1} \cdot s^{-1}$ [18]. SCN^- is a more potent inhibitor; 0.05 mM SCN^- was used so that inhibition was negligible; at these concentrations approx. 84% of $^{\cdot}OH$ radicals will react with the SCN^- taking k $(^{\cdot}OH + SCN^-) = 1.1 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ [18]. The inactivation of carbonic anhydrase in N_2O-saturated solutions at pH 7 containing SCN^- and Br^- is compared with that in N_2O-saturated solution alone (Fig. 4). For both anions the D_T value is greatly reduced reflecting the more selective nature of reactions of $(SCN)_2^{\cdot-}$ and $Br_2^{\cdot-}$ with amino acid residues compared with reactions of $^{\cdot}OH$ (see below). For solutions containing Br^- a protective effect in the exponential region (G(inact) = 0.69) is observed compared with the inactivation of $^{\cdot}OH$ (G(inact) = 1.01). Within experimental error, SCN^- has no modifying effect on the slope of this region of the inactivation curve, apparently not reflecting the more selective reactivity of $(SCN)_2^{\cdot-}$ compared with $^{\cdot}OH$. However, in the N_2O-saturated solution, exponential inactivation is only observed after a threshold dose of approx. 25 Gy. At this dose, about nine $^{\cdot}OH$ radicals will have been generated for each enzyme molecule in solution, and the enzyme will be damaged by reaction of these $^{\cdot}OH$ radicals before the exponential region of the inactivation curve is observed. In the case of inactivation by $(SCN)_2^{\cdot-}$, the threshold dose is very much less (Fig. 4), showing the greater selectivity of reactions of $(SCN)_2^{\cdot-}$ with amino acid residues of carbonic anhydrase compared with those of hydroxyl radical. The data show that carbonic anhydrase can be inactivated by one-electron oxidation of an

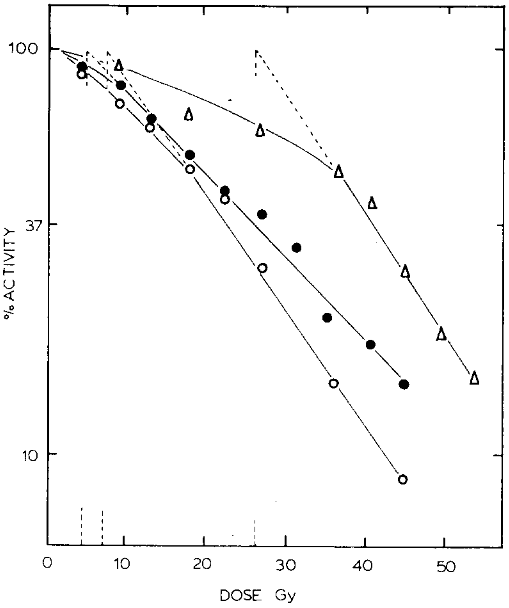


Fig. 4. Inactivation of carbonic anhydrase (0.05 mg/ml in 1 mM phosphate buffer, pH 7.0) by oxidising radicals in N_2O-saturated solutions: △, N_2O-alone; ●, containing 5 mM KBr; ○, containing 50 μM KSCN.

essential tryptophan or tyrosine residue, since $(SCN)_2^{\cdot -}$ is highly selective in its reactions with amino acids and shows only detectable reaction with tryptophan and tyrosine residues and with cysteine residues (which this enzyme does not contain) in proteins at neutral pH [27]. $Br_2^{\cdot -}$ is less selective than $(SCN)_2^{\cdot -}$ and also reacts with histidine residues. The reduced inactivation by $Br_2^{\cdot -}$ and pH 7 is consistent with the presence of essential tyrosine and tryptophan, and also implies that these radical anions are not oxidizing essential histidine residues in the enzyme, with which $Br_2^{\cdot -}$ is expected to react.

Examination of the dependence of *G*(inact) on pH (Fig. 5) shows that inactivation by both $Br_2^{\cdot -}$ and $(SCN)_2^{\cdot -}$, like that by the OH radical, depend upon the state of ionization of the activity-linked group of carbonic anhydrase. There is notably little change in *G*(inact) between pH 10 and 11, implying that ionization of the single tyrosine residue with a pK_a of 10.8 does not substantially affect the overall reactivity of these radicals with carbonic anhydrase.

Reactions of radical anions with the holoenzyme studied by pulse radiolysis. Rate constants for the reaction of $Br_2^{\cdot -}$ and $(SCN)_2^{\cdot -}$ with carbonic anhydrase

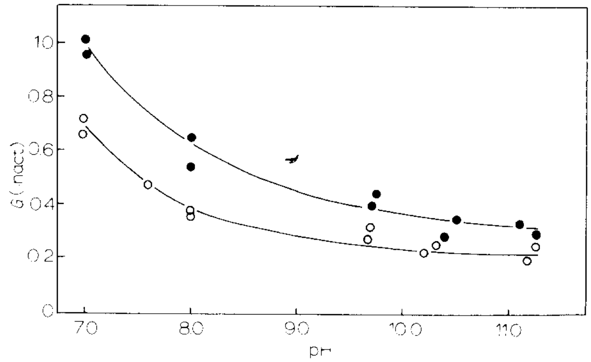


Fig. 5. Effect of pH on inactivation *G* value for N_2O-saturated solutions of carbonic anhydrase (0.05 mg/ml) containing: ●, 50 μM KSCN; ○, 5 mM KBr.

were measured at neutral and alkaline pH and are presented in Table I. The rate constant for reaction of $(SCN)_2^{\cdot -}$ with carbonic anhydrase at pH 7 ($2.2 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) is in good agreement with the value of $1.5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ obtained by Redpath et al. [5]. Redpath et al. also noted that this value is lower than that for reaction of $(SCN)_2^{\cdot -}$ with other enzymes, concluding that this reflects the known lack of exposure of tryptophan residues in the carbonic anhydrase molecule. A similar low reactivity of $(SCN)_2^{\cdot -}$ is observed in some other proteins with low tryptophan content, such as subtilisin Carlsberg [20]. The drop in rate of $(SCN)_2^{\cdot -}$ reaction with carbonic anhydrase at pH 11 to $9.5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ is unusual, as the rate constant is expected to increase with increasing pH in the region where tyrosine residues ionize. The rate

TABLE I

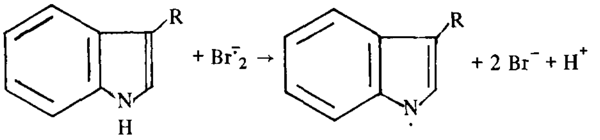
SECOND-ORDER RATE CONSTANTS FOR REACTION OF INORGANIC RADICAL ANIONS WITH HOLO- AND APO-BOVINE CARBONIC ANHYDRASE B

Temperature 22 ± 2°C, rate constants are units of $(1.0 \pm 0.1) \cdot 10^8$ $M^{-1} \cdot s^{-1}$.

Enzyme preparation	pH	Radical anion	
		$Br_2^{\cdot -}$	$(SCN)_2^{\cdot -}$
Holocarbonic anhydrase	7.0	3.2	2.3
	~11	1.9 (pH 10.8)	0.95 (pH 11.1)
Apocarbonic anhydrase	7.0	4.5	1.3
	~11	5.8 (pH 11.5)	3.2 (pH 11.1)

of $Br_2^{\cdot-}$ reaction is also unusual being only slightly higher than that for $(SCN)_2^{\cdot-}$ at neutral pH and is also reduced on increasing the pH of the solution. The lack of any increase in rate in alkaline solutions shows, as also suggested by the inactivation data, that surface tyrosine residues ionizing in the normal p*K* region and providing prominent sites of oxidative attack are not a feature of carbonic anhydrase.

The transient product spectrum from reaction of $Br_2^{\cdot-}$ with carbonic anhydrase at pH 7 is shown in Fig. 6. The spectrum contains a clear indication of the neutral tryptophan radical formed by the reaction:



with absorption maxima at approx. 320 and 520 nm. Taking the extinction at 520 nm as $1.75 \cdot 10^3$ l · mol^{-1} · cm^{-1} [28], it can be calculated that approx. 40% of radicals form the Trp residue radical. The rest must react elsewhere in the molecule, as evidenced by the peak at 400 nm in the difference spectrum (carbonic anhydrase –0.4*Gε* Trp$^{\cdot}$). This evidence shows the formation of either the histidine or tyrosine residue radicals, both of which absorb at this wavelength.

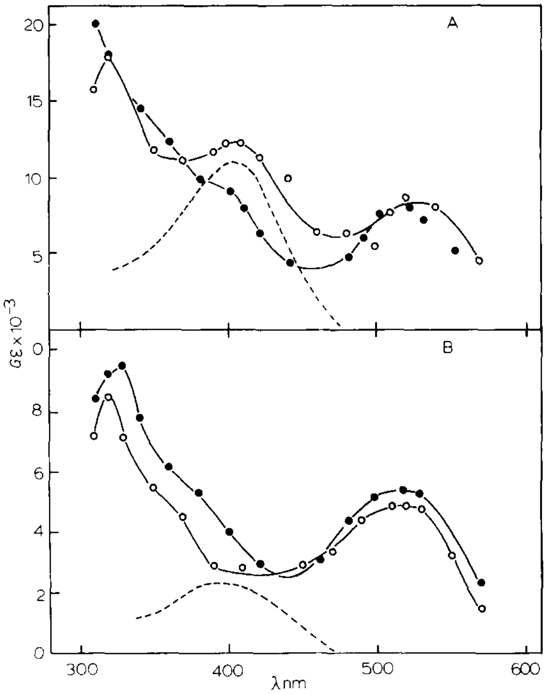


Fig. 6. Transient product spectra from pulse radiolysis of N_2O-saturated carbonic anhydrase solutions at pH 7.0. A: solutions containing 0.1 mM KSCN, corrected for $^{\cdot}OH$ radical contribution; ●, apocarbonic anhydrase (2.5 mg/ml) + 50 μM EDTA; ○, holo-carbonic anhydrase (2.5 mg/ml); dashed line = holocarbonic anhydrase spectrum minus 0.7*Gε* of tryptophan radical spectrum (measured 300 μs after pulse). B: solutions containing 10 mM KBr; ○, holo-carbonic anhydrase (1.17 mg/ml); dashed line = holo-carbonic anhydrase spectrum minus 0.4*Gε* of tryptophan radical spectrum; ●, apo-carbonic anhydrase (1.5 mg/ml) + 50 μM EDTA (measured 250 μs after pulse).

To obtain the spectrum form reaction of $(SCN)_2^{\cdot-}$ with the uninhibited carbonic anhydrase it was necessary to use a SCN$^-$ concentration of 0.1 mM. Under the conditions employed, taking the rate constants described above, only 17% of the OH radicals are scavenged by SCN$^-$, the remaining 83% reacting with the enzyme. The resulting spectrum was therefore corrected by subtracting 83% of the OH-adduct spectrum, and multiplication of the residual spectrum by 100/17 so that that intensity of the true $(SCN)_2^{\cdot-}$ derived spectrum could be compared with the $Br_2^{\cdot-}$ product spectrum. The spectrum formed from the reaction of $(SCN)_2^{\cdot-}$ with carbonic anhydrase (Fig. 6) has approx. twice the intensity at 520 nm of that from $Br_2^{\cdot-}$. The extinction at this wavelength corresponds to 70% of $(SCN)_2^{\cdot-}$ reacting with tryptophan residues. There is similarly a larger component in the calculated difference spectrum (*Gε* (carbonic anhydrase) –0.7 *Gε* (tryptophan)) at 410 nm. These features are consistent with $(SCN)_2^{\cdot-}$ being more selective than $Br_2^{\cdot-}$. Reaction of the latter also occurs with histidine, forming a weakly absorbing radical, whereas $(SCN)_2^{\cdot-}$ will react with only tyrosine and tryptophan forming the dominant peaks with larger extinction at 410 and 520 nm, respectively.

At higher pH values (approx. 10.8) the corrected spectrum of the $(SCN)_2^{\cdot-}$ oxidation product of carbonic anhydrase shows the same characteristic features as at pH 7 (Fig. 7) and, in accord with there being only one ionised tyrosine residue at this pH which is not easily accessible, there is no increase in the yield of the 410 nm peak of the tyrosine radical. In the spectrum obtained from oxidation of carbonic

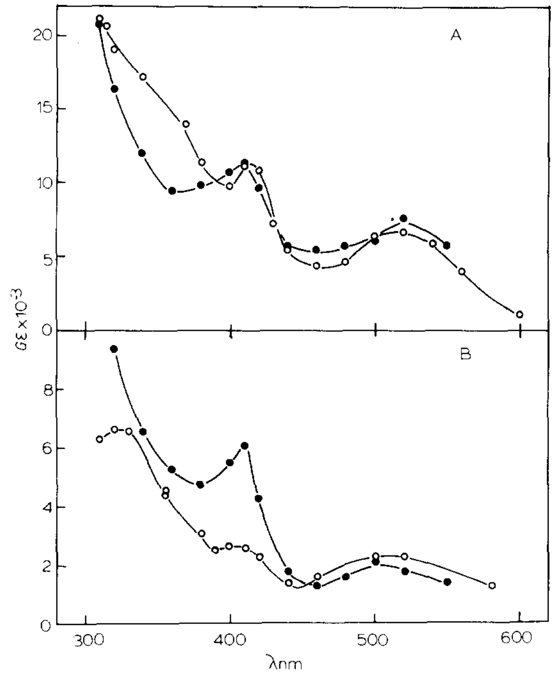


Fig. 7. Transient product spectra from pulse radiolysis of N_2O-saturated carbonic anhydrase solutions at approx. pH 11. A: containing SCN^-, ○, holoenzyme (2.5 mg/ml) + 0.1 mM KSCN, pH 10.8; ●, apoenzyme (1.32 mg/ml) + 50 μM EDTA + 0.5 mM KSCN, pH 11.1 (measured 400 μs after pulse, corrected for $^{\cdot}OH$ contribution). B: solutions containing 10 mM KBr, ○, holoenzyme (1.25 mg/ml), pH 10.5, 500 μs after the pulse; ●, apoenzyme (1.5 mg/ml), +50 μM EDTA, pH 11.15, 250 μs after the pulse.

anhydrase by $Br_2^{\cdot-}$ at pH 10.5, the extinction at 410 nm is only slightly increased over that at pH 7.0, and the 520 nm absorption due to the tryptophan radical has significantly diminished indicating that a greater proportion of $Br_2^{\cdot-}$ radicals react with histidine residues at higher pH. These spectra clearly identify tyrosine and tryptophan as the site of reaction of $(SCN)_2^{\cdot-}$ with carbonic anhydrase; $Br_2^{\cdot-}$ is seen also to react with histidine residues. A combination of these results with the inactivation data demonstrates that the inorganic radical anions inactivate carbonic anhydrase by oxidation of one or more essential tryptophan residues. Since carbonic anhydrase is less sensitive to inactivation by $Br_2^{\cdot-}$ than by $(SCN)_2^{\cdot-}$, there is no evidence for inactivation as a result of oxidation of histidine residues. Considering the role of histidine in binding Zn^{2+} at the active site of carbonic anhydrase this lack of evidence for essential histidine residues is surprising. Co-ordination of histidine to Zn^{2+} possibly lowers the electron density on the imidazole nitrogen atoms, and reduces the reactivity of these specific residues with the radical anions. Experiments are required to test this hypothesis. Inactivation of carbonic anhydrase by direct oxidation of a tyrosine residue by $Br_2^{\cdot-}$ and $(SCN)_2^{\cdot-}$ appears to be unlikely since the G value for inactivation does not increase in alkaline solutions (Fig. 5) where ionization of the single partially exposed tyrosine residue occurs with a pK of 10.8 [4]. No experimental evidence was obtained by pulse radiolysis of intramolecular free radical migration from tryptophan to tyrosine residues, as reported by Prutz et al. for other proteins [33]. Neither formation of the tyrosine radical absorption at 410 nm, nor the corresponding decay of the tryptophan radical absorption at 520 nm was seen over the first 500 μs following the radiation pulse in solutions of carbonic anhydrase containing $Br_2^{\cdot-}$ at pH 7. However, this does not eliminate the possibility of very fast radical migration occurring within the initial 100 μs time period when events are obscured by the decay of the more intense $Br_2^{\cdot-}$ absorption.

In the human B and C enzymes, two partially exposed tryptophan residues appear to occupy positions close to the active site [4]. Trp 122 of the human C enzyme is situated at the entrance to the active cavity, and Trp 207 of the human C enzyme is part of an extensive hydrogen-bonded network involving His 118 which is one of the histidine residues acting as ligand to Zn^{2+}. The amino acid sequence of the bovine B enzyme [24] shows two tryptophan residues occupying homologous positions in the polypeptide chain. One or both of these tryptophan residues in the bovine enzyme may be the crucial residue involved in the free radical inactivation process. Since all but one of the tyrosine residues are masked within the protein structure, forming part of the characteristic aromatic clusters in carbonic anhydrase, the lack of reactivity of the tyrosine residues in this enzyme is not surprising.

Reactions of radical anions with the apoenzyme studied by pulse radiolysis. A comparison was made of the reactions of $Br_2^{\cdot-}$ and $(SCN)_2^{\cdot-}$ between carbonic anhydrase and the Zn^{2+}-free apoenzyme. The rate constants at neutral and alkaline pH are included in Table I. At neutral pH, $Br_2^{\cdot-}$ reacts slightly more rapidly ($k = 4.5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) with the apoenzyme than with the holoenzyme ($k = 3.2 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) although the reaction is still slower than that between $Br_2^{\cdot-}$ and tryptophan ($k = 7.7 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ [27]). The corresponding transient product spectra from reactions of $Br_2^{\cdot-}$ with the two forms of the enzyme are similar, but that from the apoenzyme is slightly more intense. This observation shows that oxidation of tryptophan and tyrosine is favoured more in the apoenzyme and the extent of reaction with histidine residues is reduced, since the former two amino acid radicals have higher extinction coefficients than that of the latter.

$(SCN)_2^{\cdot-}$ was found to react less rapidly with the apoenzyme ($k = 1.3 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) than with the holoenzyme ($k = 2.3 \cdot 10^8$ $M^{-1} \cdot s^{-1}$). The overall shapes of the transient product spectra produced by $(SCN)_2^{\cdot-}$ are similar. Since both these spectra were substantially corrected for the $^{\cdot}OH$ contribution, due to the low SCN^- concentration used, a quantitative discussion of any differences between the apoenzyme and the holoenzyme in this instance is not justified. When compared with the rate constants for reaction of $Br_2^{\cdot-}$ and $(SCN)_2^{\cdot-}$ with free amino acids [27] at neutral pH, the rate constant data for carbonic anhydrase would indicate that in the holoenzyme reaction of $(SCN)_2^{\cdot-}$ with tryptophan and tyrosine residues is more favoured than that of $Br_2^{\cdot-}$, probably as a result of the ordered water structure around Zn^{2+}. However, with the apoenzyme the rate constants more accurately reflect the relative rates obtained with the constituent free amino acids.

In the same context, the rate constants for reaction of both radical species with the apoenzyme increase at alkaline pH, has been found with free tyrosine and non-metallo enzymes [19,20]. Conversely, an unusual decrease in rate at higher pH was noted with the holoenzyme. The spectrum formed by reaction of $Br_2^{\cdot-}$ with apo-carbonic anhydrase at pH 11.5 contains a more prominent peak at 410 nm, characteristic of the tyrosine radical, and less absorbance due to the tryptophan radical at 520 nm when compared with the spectrum obtained from the holoenzyme at pH 10.5 (Fig. 7B). The spectra obtained with $(SCN)_2^{\cdot-}$ are reasonably similar except between 320 to 380 nm, and again since these are spectra corrected for the $OH^{\cdot}$ contribution a quantitative evaluation is not justified.

The removal of Zn^{2+} from carbonic anhydrase causes no appreciable change in the conformation of the polypeptide chain, as judged from ORD spectra [7]. Slight alterations in environment of the aromatic residues (Trp and Tyr) occur on removal of Zn^{2+}, as indicated by changes in the absorption and fluorescence spectra of the enzyme [30]. The present results show that the reactivity of the same types of residues (Tyr and Trp) with radical species depends to some degree on the presence of Zn^{2+} in the active centre of carbonic anhydrase.

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