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pH Dependence Study of the Kinetic Reaction of Bovine Carbonic Anhydrase with 2,2′-Dithiobispyridine in the Absence and Presence of Surfactants

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ABSTRACT

The pH dependence study reveals that the Cys 206 sulphydryl group of bovine carbonic anhydrase in the native form is not exposed. During the reaction of 2,2′-dithiobispyridine (2-DTP) with the enzyme, there was no absorbance change recorded. In the presence of surfactants, the pH dependence profiles of the apparent second order rate constants, k_{app}, for the reaction of 2-DTP with bovine carbonic anhydrase assume simple profile forms, which resemble the diprotic acid titration curve. These simple profiles were quantitatively analyses with Eq. (1) in the text. Best-fit lines drawn with the parameters shown in Table 1 are obtained. The mean pK_1 value of 5.1 and the mean pK_2 of 8.4 are assigned to His 167 and Cys 206, respectively. The pH dependence of the apparent second-order rate constant, k_{app}, in the higher concentrations of SDS is higher than that in the lower concentrations. It shows that the Cys 206 sulphydryl group of bovine carbonic anhydrase is more exposed in the higher concentrations of SDS.

Keywords: Bovine Carbonic anhydrase; Sulphydryl reactivities; pH; Surfactants

INTRODUCTION

Carbonic anhydrase (CA, carbonate hydrolyase, EC 4.2.1.1) catalyses the inter-conversion of carbon dioxide and bicarbonates. The enzyme has a roughly spherical structure with the active site comprising a conical cleft about 15 A° deep. One side of the cavity is formed by hydrophobic residues and the other side contains hydrophilic residues including Thr 199 and Glu 106. The zinc ion is located at the bottom of this cleft, and tetrahedrally coordinated to the imidazoles of the three histidines residues (His 94, His 96 and His 119) and to a water molecule (called the "zinc water") that ionizes to a hydroxide ion with a pK about 7[1~3]. Amino acid residues Thr 199 and Glu 106 contribute to a hydrogen-bonding network with the Zn-OH^- that maintains the catalytically competent structure optimal for nucleophilic attack by the zinc-bound hydroxide on the CO_2 substrate [4,5].

A lot of work has been done on the structural and conformational changes of carbonic anhydrase using surfactants or other methods of structural elucidation[1~5] but there is no reported case of quantitative analysis of sulphydryl reactivities of bovine carbonic anhydrase with any sulphydryl reagents to the best of our knowledge.

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Bovine carbonic anhydrase has only one cysteine residue located in the wrapping domain at position 206[1]. Detailed investigations of the chemistry of the reaction of the 2, 2'-dithiobispyridine (2-DTP) with cysteine, thiol compounds and hemoglobin have been carried out[6~8]. The uncharged reagent, 2-DTP, is relatively unaffected by the charges on the protein and it is primarily influenced by changes in the pK of the thiol group or by the tertiary and quaternary structural changes in the protein. The pKs of thiol groups are known to be sensitive to neighboring titratable groups[9]. We decided to select 2-DTP to study the sulphydryl reactivities of bovine carbonic anhydrase because the outer side is covered by hydrophobic residues. In this paper we have undertaken a comprehensive pH-dependence study of the kinetics of reaction of bovine carbonic anhydrase with 2-DTP in the absence and presence of surfactants. The 2-DTP was simpler and was quantitatively analyzed. Our analysis shows that two ionizable groups are coupled to the reaction of the sulphydryl group: Cys 206 and His 167.

MATERIALS AND METHODS

Bovine carbonic anhydrase and 2-DTP were purchased from Sigma Chemical Company and were used without further purification.

2-DTP Kinetics: The concentration of 2-DTP was determined by dissolving a known weight in 95% absolute ethanol. An aliquot of this solution was taken up in phosphate buffer (pH. 7.0, ionic strength 0.2 M) and its concentration was determined spectrophotometrically at 281 nm using an extinction coefficient of 9730 M^{-1} cm^{-1}[9]. The kinetic experiments were performed as follows: The protein solution were prepared in phosphate buffer (pH 6-8) and borate buffer (pH>8) and of total ionic strength of 0.05 M. A 5 μM bovine carbonic anhydrase solution in a chosen buffer was allowed to equilibrate at 27°C in a thermostat for almost 3 hours. A 3 ml of this solution were then transferred to a 1cm×1cm cuvette. The cuvette was placed in the cell compartment of a Shimadzu temperature controlled cell holder TCC-240A computerized double beam UV-3100 spectrophotometer at 27°C. A few microliters of 2-DTP of known concentration (100 μM) was measured with a finn pipette into a glass rod shaped in a shallow spoon form. The rod was used to add the 2-DTP and to stir the 2-DTP-Carbonic anhydrase mixture. The absorbance of the mixture was recorded as a function of time at a wavelength of 343 nm by the spectrophotometer. Each kinetic run was repeated twice under identical experimental conditions. In the presence of ionic surfactants, the concentrations of SDS used are 0, 0.04 mM, 0.2 mM and 5 mM while that of DTAB is 12 mM. Apparent second order rate constant, k_{app}, was calculated with the second-order rate equation. The extinction coefficient used in the calculation at 27°C had been reported for 2-thiopyridone [9], which is the product of 2-DTP reaction. The extinction coefficient of 2-thiopyridone determined was 7.56 $mM^{-1}cm^{-1}$ and independent of pH and temperature. The pH of the reaction mixture was determined using Beckman 0TM50 pH/ISE meter. The software used for fitting the data was TableCurve2D v^{50}. All experiments were carried out at constant temperature of 27°C.

RESULTS AND DISCUSION

It is known[10] that the rate of hydrolysis of disulphide bonds increases markedly above pH 9. Therefore, to avoid any uncertainty about the integrity of the 2-DTP reagents, we restricted our studies to the region below pH 9. Consequently, we were unable to work at the high pH region where k_{app} should "level off" to give values of limiting second-order rate constants. The emphasis in this paper is to obtain the pK_a values of ionizable groups responsible for the sulphydryl reactivity.

In the native form, there was no absorbance change recorded which confirms that the Cys 206 is not exposed to the water surface. It also confirms earlier reports that the outer chain of CA moiety is covered by some hydrophobic side chains[1~3]. 2-DTP is a

neutral sulphydryl reagent. Due to the fact that some hydrophilic chains are buried within the enzyme, it hinders the accessibility of Cys 206 to the sulphydryl reagent. The tertiary structure of CA shows that Cys 206 at the wrapping domain is shielded from the surface by the α-helix, β7 and β8 sheets, which also contribute significantly to the unexposure of the sulphydryl group of CA[1]. In the presence of SDS, in the lower concentrations, the CA moiety was partially exposed but the little absorbance changes were recorded at 0.04 mM and 0.2 mM SDS. It shows that 2-DTP has access to the binding site of Cys 206 of CA. The pH dependence study of the rate of reaction of Cys 206 of CA with 2-DTP in the presence of SDS suggests that the rate of unfolding in anionic surfactant is modulated by repulsion between surfactant head group and anionic side chains[11]. Fig. 1 shows the plots of k_{app} versus pH at 0.04 mM and 0.2 mM of SDS. The pH dependence study in the presence of SDS shows that the second-order rate constants, k_{app}, increase with increasing pH in a titrable fashion. This change could be attributed to conformational changes exhibited by the CA at each pH. It could also be assumed that the total charge of the ionizable side chains on the enzyme would vary significantly. It is not unlikely that several rapid conformational changes are taking place but it is not possible to monitor them under present experimental conditions. Our focus is to study the sulphydryl activities of CA in the presence of SDS at low concentrations. In Fig. 1, the pH dependence profiles of the apparent second-order rate constant, k_{app}, for the reaction of CA assume simple forms resembling the titration curve of a diprotic acid. These curves are similar to our previous findings in other proteins[8,12]. We have previously accounted for profiles similar to those in Fig. 1 in terms of the fractional population of the thiol anion form of the sulphydryl group and histidine residue. This consideration led to the equation[8,12]:

$$k_{app}=k_1 \frac{K_1}{K_1+[H^+]} \quad \frac{K_2}{K_2+[H^+]}+k_2 \quad (1)$$

In eq. (1), k_1 is the limiting apparent second-order rate constant for the 2-DTP reaction when the reactivity of the Cys 206 is electrostatically linked to the ionization of the His 167, with ionization constant, K_1; k_2 is the limiting apparent second-order rate constant at high pH when the sulphydryl reagent is linked to the ionization of Cys 206 with ionization constant, K_2. The first fractional term is the fraction of the neutral form of histidine, while the second fractional term is the fraction of the thiol anion form of the sulphydryl.

Analysis of the data shown in Fig. 1 using eq. (1) gave the best fit parameters as shown in Table 1.The curves in Fig 1 are theoretical best-fit lines calculated using these parameters. Very good fits are obtained for all the data. For the 2-DTP reaction at 0.2 mM SDS, the value of pK_1 is 4.9 and the value of pK_2 is 8.1. We assigned these pK_a values to His 167 and Cys 206. These analyses are similar to the reported parameters published before[8,12]. The low values of the pK_a when compared to our previous findings show that the amino acid residues are in dynamic equilibrium between the internal and external sphere.

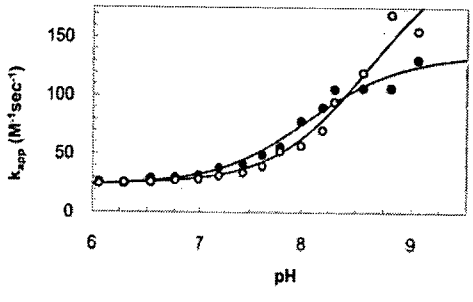

Fig.1. Dependence of k_{app} on pH for the reaction of 2-DTP with the cys 206 sulphydryl group of bovine carbonic anhydrase at ionic strength 0.05 M at 27°C. 0.04 mM SDS, ○ and 0.2 mM SDS, ●. [CA]=4 μM and [2-DTP]= 40 μM. The lines drawn are theoretical best-fit lines calculated from eq. (1) using the parameters given in Table 1.

In the higher concentration of SDS, the pH dependence of the apparent second-order rate constants, k_{app} at 5 mM SDS are higher in Fig 2 than Fig 1. It shows that the CA moiety has been exposed. It further reveals that ability to unfold proteins stems from the amphiphilic properties shared by protein and surfactant. For example, SDS binds to CA via interaction between the sulfate group and positively charged amino acid side chains and between the alkyl chain and hydrophobic side chains[11]. Since Cys 206 and His 167 are buried in the native form of CA, high concentration of SDS unfolds partially the hydrophilic side chains to the water solvent. The rate of reaction is faster with CA in 5 mM SDS than in 0.04 and 0.2 mM SDS. It should be noted again that 2-DTP is a neutral sulphydryl reagent. It is unaffected by the charges on the ionizable groups of the enzyme.

Fig. 2 was quantitatively analyses using eq. (1), which gave the best-fit parameters in Table 1. The curve in Fig 2 is a theoretical best-fit line calculated using these parameters. Very good fit is obtained. The pK_1 value for His 167 is 4.9 while the pK_2 value for Cys 206 is 8.6. The pK_1 values obtained at various SDS concentrations are the same which confirms that the His 167 is in the same hydrophobic environment. In Table 1, the pK_1 values of His 167 are similar, therefore, SDS does not unfold the positively charged amino acid residues. The pK_2 values in the range of SDS concentration differ slightly which shows that the Cys 206 sulphydryl group is "almost" in the same environment. The pK_2 value at 0.2 mM SDS is lower than compare with the pK_2 value at 5 mM SDS as shown in Table 1. It shows that at lower SDS concentration, the Cys 206 is in dynamic equilibrium between the inner and outer sphere while it is exposed in higher SDS concentration.

DTAB is a cationic surfactant. In DTAB the cationic side chains modulate folding of the enzyme. It binds with the anionic amino acid side chains of the enzyme.

The only reported case of DTAB in this experiment is at a higher DTAB concentration. Our aim is to unfold completely the protein. Fig. 3 shows the pH dependence profile of the apparent second-order rate constants in the presence of DTAB.

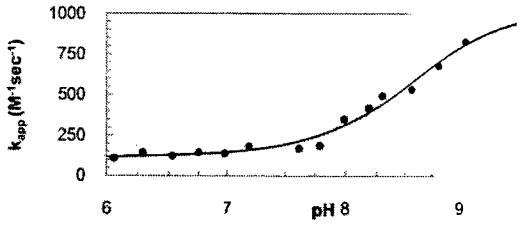

Fig.2. Dependence of k_{app} on pH for the reaction of 2-DTP with the cys 206 sulphydryl group of bovine carbonic anhydrase at ionic strength 0.05 M at 27°C. [CA]=4 μM, [SDS]=5 mM and [2-DTP]=40 μM. The line drawn is theoretical best-fit line calculated from eq. (1) using the parameters given in Table 1.

The profile resembles a diprotic acid titration profile. Fig. 3 was analyzed with eq. (1). The curve in Fig 3 is a theoretical best fit line calculated using these parameters. Equation 1 gave best-fit parameters as shown in Table 1. The pK_1 value for His 167 is 5.5 and pK_2 value for Cys 206 is 8.4. The pK_1 and pK_2 values are in perfect agreement with our previous findings [8,12]. So it seems that His 167 and Cys 206 are electrostatically linked to the sulphydryl reactivities of CA.

We conclude that anionic surfactant does not alter the pK_a value of the positively charged amino acid residues because these groups are in a more hydrophilic environment while cationic surfactant alters the pK_a of some ionizable groups, which subsequently influence the pK_a of histidine. Finally, the Cys 206 sulphydryl group on the wrapping domain alternates between the internal and external conformation of the enzyme. Since bovine carbonic anhydrase II has not yet been crystallographied, these data can create some sight view to the tertiary structure of the enzyme.

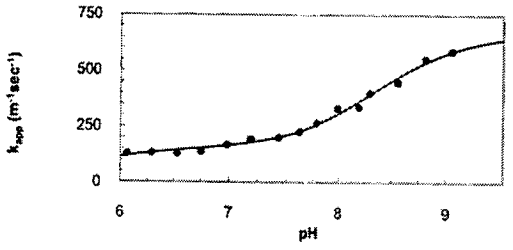

Fig.3. Dependence of k_{app} on pH for the reaction of 2-DTP with the Cys 206 sulphydryl group of bovine carbonic anhydrase at ionic strength 0.05 M at 27°C. [CA]=4 μM, [DTAB]=12 mM and [2-DTP]=40 μM. The line drawn is theoretical best-fit line calculated from eq. (1) using the parameters given in Table 1

Table 1: Kinetic parameters of the reaction of 2-DTP with the Cys 206 sulphydryl group of bovine carbonic anhydrase at 27°C.

	pK_1	pK_2	k_1 ($M^{-1}s^{-1}$)	k_2 ($M^{-1}s^{-1}$)
Native	—	—	—	
0.04 mM SDS	4.9±0.2	8.6±0.2	24.0±0.2	1138±0.2
0.2 mM SDS	4.9±0.2	8.1±0.2	24.2±0.2	1114±0.2
5 mM SDS	4.9±0.2	8.6±0.2	122.0±0.2	917.2±0.2
12 mM DTAB	5.5±0.3	8.4±0.3	149.5±0.3	527.7±0.3

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