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THE REACTION OF 5,5-DITHIOBIS(2-NITROBENZOIC ACID) WITH BOVINE LIVER CATALASE WITH MULTIPLE REACTIVE SULPHYDRYL GROUPS

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ABSTRACT. Catalase has sixteen sulphydryl groups per tetramer located at the α-helix Cys-459, ß-sheet Cys-231, wrapping domain Cys-376 and wrapping domain Cys-392. The sulphydryl group of denatured bovine liver catalase are reactive towards non-mercurial sulphydryl reagent, 5,5-dithiobis(2-nitrobenzoic acid).

We have investigated the kinetics of the reaction of 5,5-dithiobis(2-nitrobenzoic acid), DTNB, with bovine liver catalase as a function of pH in the presence or absence of sodium n-dodecylsulphate (SDS). In the native form, there was no change in absorbance which shows sulphydryl groups of catalase are not exposed to the solvent. In the denatured form, the reaction was monophasic. Quantitative analysis of the pH dependence of the apparent second order rate constant reveals that the thiolate anion is the modulating factor. The pK_a is assigned to the sulphydryl group of catalase.

INTRODUCTION

Catalase (EC:1.11.1.6; H_2O_2 oxidoreductase) is one of the most intensively studied of all mammalian enzymes [1-12]. Bovine liver catalase is typically a tetramer of identical heminic subunits (MW 240,000). X-ray studies have revealed the detailed structure of bovine liver catalase [1-3]; and its amino acid sequence was determined [4]. Catalase has 16 sulphydryl groups located at the following positions: α-helix Cys-459, β-sheet Cys-231, wrapping domain Cys-376 and wrapping domain Cys-392 [3].

We have carried out a comprehensive pH dependence study of the reaction of 5,5-dithiobis(2-nitrobenzoic acid), DTNB, with catalase in the presence and absence of SDS. In this paper the determination of the pK_a values of the sulphydryl group will be discussed.

MATERIALS AND METHODS

Crystalline bovine liver catalase (obtained as a suspension in water containing 0.1% thymol) and DTNB were purchased from Sigma Chemical Co. Ltd. and were used without any further treatment. SDS (especially pure grades) was also supplied by Sigma.

DTNB solution was prepared by dissolving 0.9907 g of DTNB in phosphate buffer (pH 7.6) of ionic strength 0.05 M and the mixture was stirred for four days. The solution was then filtered and its concentration was determined spectrophotometrically by measuring the absorbance at 412 nm after reaction in excess of 2-mercaptoethanol; a molar extinction coefficient of 13,600 $M^{-1}.cm^{-1}$ was assumed for 3-carboxylato-4-nitrothiophenolate [3].

Kinetic studies were conducted on a Shimadzu computerized double beam UV-160 spectrophotometer at 27°. Solutions of catalase (5 μM) were prepared in phosphate buffers (pH 6.0 to 8.0) and borate buffers (pH 8), each of total ionic strength of 0.05 M. The solutions were allowed to equilibrate at the desired temperature. The kinetics of the reaction of DTNB with catalase in the presence of SDS was carried out by dissolving a known amount of SDS in each buffer solution. Catalase (5 μM) was added to each buffer solution containing SDS and the solutions were allowed to equilibrate for a period of 1 h.

The kinetics of the reaction of DTNB with catalase in the absence of SDS was carried out by pipetting 1 mL aliquot of the solution into a 1 x 1 cm cuvette which was subsequently placed in the cell compartment of the spectrophotometer. A few microlitres of DTNB (30 μM) was measured with a Finn pipette into a glass rod shaped in a shallow spoon form. The rod was used to add the DTNB solution and to stir the DTNB catalase mixture. There was no change in absorbance as a function of time at a wavelength of 412 nm. The same procedure was repeated for the reaction of DTNB with catalase in the presence of SDS. Each kinetic run was repeated twice under identical experimental conditions. The number of sulphydryl groups that reacted with DTNB was determined using the Boyer method [17].

The first order plots were resolved by graphical method. The graphical methods were used primarily to obtain the initial estimates of some of the rate constants which were further refined by least-squares analysis of the reaction time course. First order rate constants were calculated from the slopes of ln (A_∞ - A_t) *vs* time plots, A_∞ = absorbance at infinite time; A_t = absorbance at time t. The standard error in the determination of k_{obs} was about 5%. Apparent second order rate constant, k_{app}, were obtained by dividing the k_{obs} values with the DTNB concentration. The concentration of SDS used were 2.5 and 10 mM.

RESULT AND DISCUSSION

Titration of catalase with DTNB, after removing the heme groups by the acetone - HCl method, showed that this apocatalase has 10 sulphydryl groups per tetramer [9]. It has been reported in the literature that denatured apocatalase, using 8 M urea, has 14 sulphydryl groups after titrating it with sulphydryl reagent [10] and that two of them are buried inside the molecule [10].

In the present work, the titration of catalase with DTNB has been limited to pH 9 because at higher pH there would be increased rate of hydrolysis of the disulphide bonds.

The reaction of DTNB with native catalase as a function of pH was not reported because there was no change in the absorbance during the course of the reaction. This shows that the sulphydryl groups of the native catalase are not exposed to the solvent. In the presence of SDS the reaction was monophasic (Figure 1). This suggests that the sulphydryl groups on the wrapping domain must have reacted with DTNB at same rate. There are 16 sulphydryl groups in catalase located at the following positions: α-helix Cys-459, ß-sheet Cys-231, wrapping domain Cys-375 and wrapping domain Cys-392 [3]. There are three features in quaternary structure of catalase namely: α-helix, ß-sheets and wrapping domain [3]. The α-helix and ß-sheets are more stable than the wrapping domain due to the formation of hydrogen bonds among the amino acid residues. It has been reported that in the presence of denaturant, the wrapping domain is more exposed to the solvent than that of α-helix and ß-sheet [15]. From this result, the reaction is monophasic which confirms that the sulphydryl groups on the wrapping domain, i.e., Cys-376 and Cys-392 react at the same rates. From the X-ray study,

we suggest that Cys-231 must be buried within the molecule because it is located at the side of the heme-environment.

There is an electrostatic linkage between Cys-459 and His-74, and Cys-459 also senses the influence of His-210 [3]. SDS is an anionic surfactant which interacts with positively charged amino acid residues. It means that His-74 and His-210 must have interacted with SDS, which subsequently make Cys-459 not accessible to DTNB because of the SDS-catalase complexes formed around Cys-459 [3].

Figure 2 shows the linear plot of ln (A_∞ - A_t) against time. The k_{obs} for the reaction was calculated from the slopes in the presence of SDS at pH 7.6.

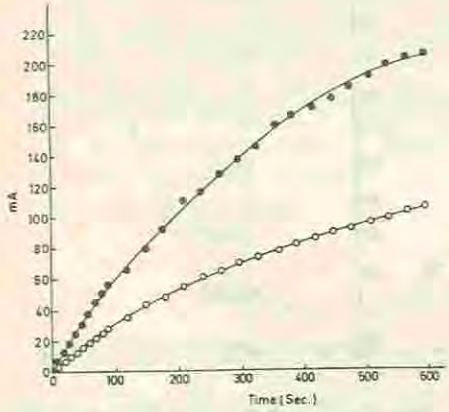

Figure 1. Fractional change (mA) *vs* time plot for the reaction of DTNB with denatured bovine liver catalase, 5.0 μM catalase before mixing in 0.05 M, phosphate buffer, pH 7.8 (o = 2.5 mM SDS and • = 10 mM SDS).

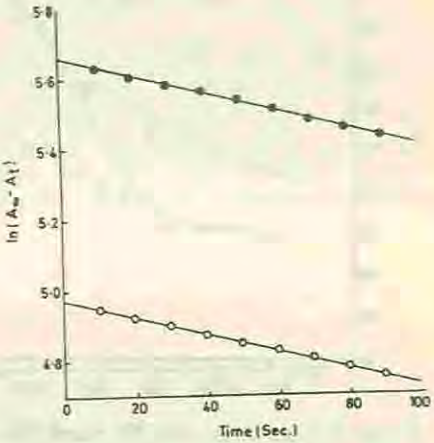

Figure 2. First order kinetic plots for the reaction of DTNB with denatured catalase in 0.05 M phosphate buffer, pH 7.6 DTNB and catalase concentrations before mixing are 30 and 5.0 nM, respectively (o = 2.5 mM SDS and • = 10 mM SDS).

Figures 3 and 4 show the apparent second order rate constants for the reaction of DTNB with catalase in the presence of 2.5 mM and 10 mM SDS, respectively. Partial denaturation occurs at SDS concentration of 2.5 mM while 10 mM is required for complete denaturation. The reaction was monophasic and slow. There were differences in the relative amplitudes of the reaction in 2.5 and 10 mM SDS. These reflect the differences in the number of SDS-catalase complexes formed and also the exposure of the reactive centers. The apparent decrease observed in the reaction rates of catalase with DTNB must have resulted from the interaction of positively charged amino acid residues with SDS (Figures 3 and 4). The reaction could have been very fast if the positively charged amino acid residues have not interacted with SDS. The critical micelle concentration (CMC) of SDS for catalase is about 10 mM [7].

Figures 3 and 4 were analyzed using Equation 1.

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

where k_1 is limiting apparent second order rate constant at the pH when the thiolate anion is the modulating factor with ionization constant K_1.

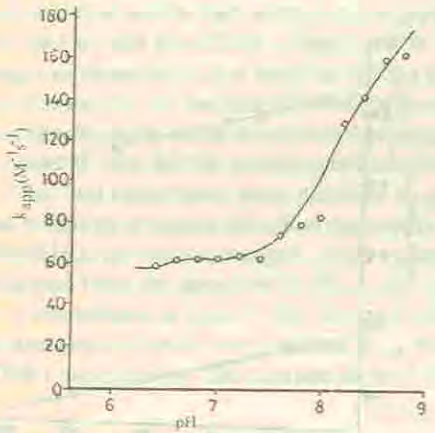

Figure 3. Comparisons of SH reactivities of denatured catalase with DTNB. Conditions: 5 μM catalase, phosphate and borate buffer with ionic strength 0.05 M, 2.5 mM SDS and 27°.

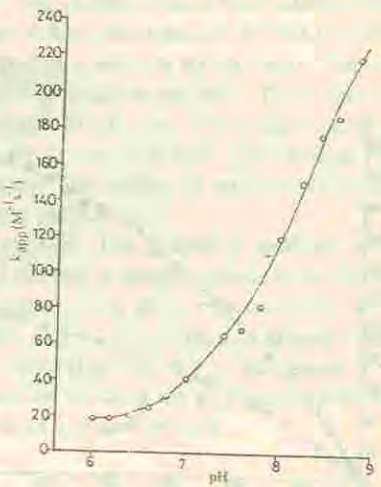

Figure 4. Comparisons of SH reactivities of denatured catalase with DTNB. Conditions: 5 μM catalase, phosphate and borate buffer with ionic strength 0.05 M, 10 mM SDS and 27°.

Figures 3 and 4 show the fit of the k_{app} *vs* pH data for 2.5 and 10 mM SDS, respectively. The fitting parameters are shown in Table 1. An examination of Table 1 shows that, for those data best fitted by Equation 1, the pK for the two sulphydryl groups of catalase in the two concentration are similar. The pK_a values of the sulphydryl group of free cysteine and glutathione in water are 8.3 and 8.75, respectively [6]. In the present work, the pK_1 is assigned to sulphydryl group of catalase.

Table 1. pK_1 and k_1 values for the reaction of DTNB with bovine liver catalase.

	2.5 mM SDS	10.0 mM SDS
pK_1	8.5	8.5
k_1 ($M^{-1}.s^{-1}$)	236.71	262.47

Generally, SDS-catalase complexes are formed within the pH range of 6.0 - 7.2 than at higher pH which might be attributed to the pattern observed in the pH - k_{app} profiles (Figures 3 and 4).

The titration of denatured catalase with DTNB at 412 nm using Boyer method [17] shows that there are eight sulphydryl groups that have reacted (Figure 5). Boyer method further confirms that Cys-376 and 392 are responsible for the monophasic reaction of DTNB with catalase.

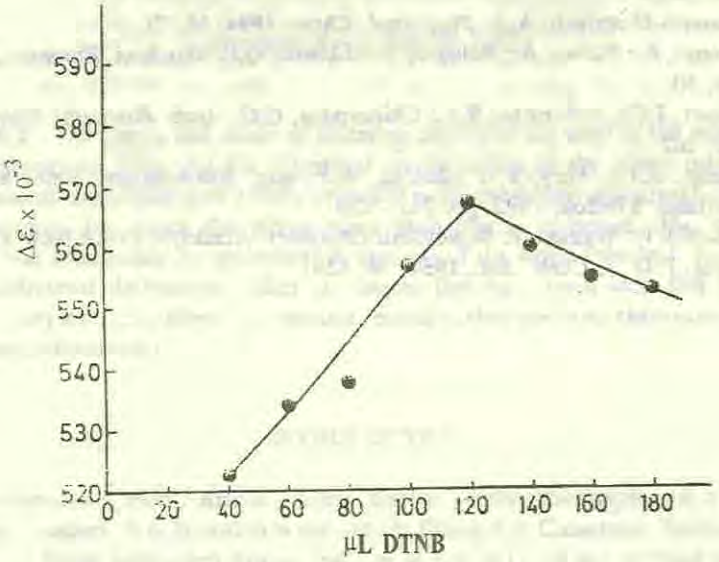

Figure 5. Plot of $\Delta\epsilon$ at 412 nm *vs* volume of 5 μM DTNB.

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