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Enthalpy investigation for elucidation of the transition concentration for the interaction of horseradish peroxidase with surfactants

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Abstract

The interaction of horseradish peroxidase (HRP) with sodium *n*-dodecyl sulphate (SDS) as an anionic detergent and *n*-dodecyl trimethylammonium bromide (DTAB) as a cationic detergent was studied at pH 6.4 by microcalorimetry, equilibrium dialysis and spectroscopy. The enthalpy of unfolding of the HRP by surfactants was determined from the calorimetric enthalpy and the enthalpy of binding was calculated from the Wyman and van't Hoff relations.

The transition concentration, $[S]_{1/2}$, for the denaturation of HRP by SDS and DTAB is enlightened from the enthalpies of unfolding and of binding. Here, it has signified that the $[S]_{1/2}$ point occurred at the end of HRP-surfactant electrostatic and the start of hydrophobic interactions. The unfolding of protein is mostly completed at the end of electrostatic interactions. © 1997 Elsevier Science B.V.

Keywords: Horseradish peroxidase; Surfactants; Anionic detergent; Cationic detergent

1. Introduction

Horseradish peroxidase (HRP, donor: H_2O_2 oxidoreductase EC 1.11.1.7) catalyses the oxidation of a wide variety of aromatic donor molecules with hydrogen peroxide [1]. HRP as a monomer enzyme with a molecular weight of 40 500, consists of an apoenzyme which contains both carbohydrate and protein, combined with an iron protoporphyrin IX as a non-covalently bound prosthetic group [2]. Its secondary structure with about 300 residues contains a large percent of α-helical conformation [3].

The interaction of surfactants with globular protein provides additional information on the structure, properties and function of protein molecule. The nature of such type of interaction involves a combination of ionic and hydrophobic interactions [4–9]. We have previously reported studies on the isothermal chemical denaturation of HRP with urea and guanidine hydrochloride, thermal denaturation of HRP and binding studies of HRP with benzhydroxamic acid [10–12].

In the present paper, we report a study of the interaction of ionic surfactants with HRP in order to obtain additional information for the peroxidase structure especially elucidation of the nature of transition concentration point for denaturation by surfactants; $[\text{surfactant}]_{1/2}$.

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2. Experimental

2.1. Materials

HRP type II with a purity index RZ ≅ 2.3, SDS, DTAB, orange II and rosaniline hydrochloride were obtained from Sigma and guaiacol was purchased from Fluka. All materials and reagents were of analytical grade. Visking membrane dialysis tubing (molecular weight cut off 10 000–14 000) was obtained from SIC (East Leigh) Hampshire, UK. All solutions were prepared in phosphate buffer, pH 6.4, $I = 0.0069$ M.

2.2. Methods

2.2.1. Microcalorimetry

The calorimetric enthalpy measurements were performed at 27.000 ± 0.005°C using LKB microcalorimeter (Thermal Activity Monitor 2277, Thermometric Sweden). The microcalorimeter is interfaced with an IBM PS/2 model 40–486 computer, and the software used is the thermometric DIGITAM 3 program. For the determination of the enthalpy, titration microcalorimetry was used with the surfactant solution in the syringe and HRP in the sample cell. The concentration of surfactant in the syringe was 10 mM and the concentration of HRP in the cell was 0.02% (w/v). The volume of HRP solution in the cell was 1 ml and the injection of surfactant volume in each step was 30 μl. During the titration, the enthalpy of demicellisation and dilution of surfactant is corrected by measuring the change due to injection of surfactant solution to buffer solution. Moreover, the heat of dilution of HRP was negligible. The microcalorimeter was electrically calibrated during the course of the study at regular intervals.

2.2.2. Equilibrium dialysis

Equilibrium dialysis was carried out at 27 and 37°C. 1 ml of HRP solutions of 0.03% (w/v) were placed in dialysis bags and equilibrated with 2 ml aliquots of detergent solution covering the required concentration range for over 96 h, which is explained previously [4]. All the measurements reported refer to DTAB and SDS concentrations below their critical micelle concentrations (CMC). The CMC of SDS and DTAB were obtained conductometrically and are 7.6 and 12.5 mM at pH 6.4, respectively [13]. The free surfactant concentrations at equilibrium with the complexes were assayed spectrophotometrically by the rosaniline hydrochloride method for SDS and the orange II method for DTAB [14,15]. Absorbance profiles for interaction of SDS and DTAB with HRP were obtained at pH 6.4 by a UV/VIS Milton Roy spectrophotometer model 3000. In all calculations the molecular weight of HRP was taken to be 40 500 [16].

The experiments were repeated thrice and the results had a good correlation.

3. Results and discussion

Fig. 1 shows the binding isotherms (the plot of $\bar{\nu}$ as the number of moles of surfactants bound per mole of enzyme vs. logarithm free concentration of surfactant) for HRP-surfactant interactions at pH 6.4 and temperatures of 27 and 37°C. Analysis of these data, obtained from equilibrium dialysis, according to the Hill equation indicates two sets of binding sites. The Hill equation for two sets of binding sites with interaction between them could be written as [17]:

$$\bar{\nu} = \frac{g_1(K_1[S]_{free})^{n1}}{1 + (K_1[S]_{free})^{n1}} + \frac{g_2(K_2[S]_{free})^{n2}}{1 + (K_2[S]_{free})^{n2}} \quad (1)$$

It is generally accepted that binding of surfactant molecules to proteins occurs by a combination of ionic and hydrophobic interactions [18,19]. So we can consider the first and second terms of Eq. (1) due to the electrostatic and hydrophobic contribution

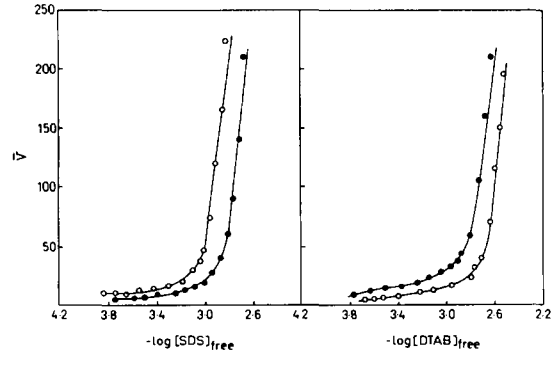

Fig. 1. Binding isotherms for HRP-surfactant systems at pH 6.4. • 27°C and ○ 37°C.

Table 1
Parameters derived from Eq. (1) for binding of surfactants to horseradish peroxidase at pH 6.4 and 27°C. Maximum errors obtained from the EUREKA, a data fitting software

t (°C)	g_1		g_2		n_1		n_2		K_1 (M^{-1})		K_2 (M^{-1})	
	SDS	DTAB	SDS	DTAB	SDS	DTAB	SDS	DTAB	SDS	DTAB	SDS	DTAB
27	22	45	220	230	2.61	1.77	9.46	8.10	2076	3040	513	497
S.D [a]	±1	±2	±5	±5	±0.12	±0.10	±0.20	±0.20	±2	±3	±1	±1

[a] Standard deviation.

in binding, respectively [20]. g_1 and g_2 are the number of electrostatic and hydrophobic binding sites, K_1 and K_2 the electrostatic and hydrophobic binding constants, and n_1 and n_2 are the Hill coefficients for electrostatic and hydrophobic contributions in the binding process, respectively. In order to fit the data to the Eq. (1), the values of g_1 and g_2 were estimated and then the binding data were fitted to this equation using a non-linear least squares program [21]. The results are listed in Table 1. The uncertainties in Table 1 show the maximum errors obtained from the fitting program.

Fig. 2 shows the sigmoidal profiles for HRP denaturation by surfactants. The upper axis in Fig. 2 represents $\bar{\nu}$ values for SDS and DTAB.

The transition concentration $[S]_{1/2}$ as the mid-point of the curve was discussed for urea and guanidine hydrochloride on the basis of the Pace model [19]. The sigmoidal absorbance profile for protein denaturation

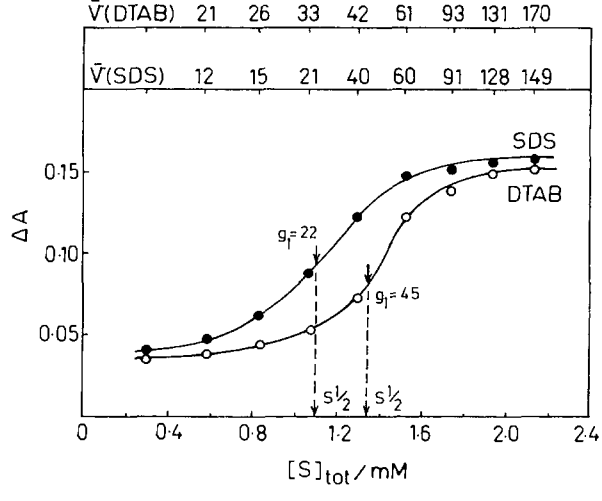

Fig. 2. Absorbance profile for SDS and DTAB on the interaction with HRP at pH 6.4 and 27°C. • SDS and ○ DTAB.

by urea and guanidine hydrochloride or temperature was first introduced by Pace and signified $[S]_{1/2}$ as the mid-point of denaturation curve (concentration of denaturant which is brought half of the protein denaturation) [22,23]. Such studies on denaturation of proteins by surfactants were at first done by Moosavi-Movahedi et al. [24]. These curves show the coincidence of $[S]_{1/2}$ as the mid-point or transition point for denaturation profile. At this point the free energy change is equal to zero, therefore $[S]_{1/2}$ is the transition point for protein denaturation with a two-state mechanism [25]. g_1 is the total number of electrostatic binding sites which coincidents with $[S]_{1/2}$ for peroxidase assuming two-state mechanism. Therefore, $[S]_{1/2}$ seems to be a saturation point for electrostatic interaction. Of course, this coincidence will be applicable for denaturation of other protein–surfactant complexes through the two-state mechanism.

The values of g_1 at 27°C and pH 6.4 for SDS and DTAB are 22 and 45, respectively (see Table 1). Also, the transition concentrations under the same conditions for SDS and DTAB are 1.10 mM and 1.35 mM, respectively (see Fig. 2). The enthalpy of binding (ΔH_{bin}) and calorimetric enthalpy (ΔH_{cal}) will be used to interpret the $[S]_{1/2}$ for HRP-surfactant interaction subtlety.

Binding isotherms were analysed based on the Wyman binding potential concept [26]. The binding potential is calculated from the area under the binding isotherms according to the equation

$$\pi = RT \int_{\bar{\nu}_{i=0}}^{\bar{\nu}_i} \bar{\nu} d(\ln[S]_{free}) \quad (2)$$

and is related to an apparent binding constant, K_{app}, as follows [27]:

$$\pi = RT \ln(1 + K_{app}[S]_{free}^{\bar{\nu}_i}) \quad (3)$$

The Gibbs free energy change associated with the binding process may be written as:

$$\Delta G^0 = -RT \ln K_{app} \quad (4)$$

The enthalpies of binding (ΔH_{bin}) were obtained from the temperature dependence of the equilibrium binding constant using the van't Hoff relation [28]:

$$\Delta H_{bin} = -R \frac{d(\ln K_{app})}{d(1/T)} \quad (5)$$

The binding enthalpies are also determined from measurements of the overall enthalpy of denaturation by microcalorimetry. The overall enthalpy could be written as follows [29]:

$$\Delta H_{cal} = \Delta H_{bin} + \Delta H_{unf} \quad (6)$$

where ΔH_{cal}, ΔH_{bin} and ΔH_{unf} are calorimetric enthalpy, enthalpy of binding, and enthalpy of unfolding, respectively. By substituting the values of ΔH_{bin} and ΔH_{cal} in Eq. (6), enthalpies of unfolding may be obtained. Figs. 3 and 4 show the changes of enthalpy values as a function of total concentration of SDS and DTAB, respectively. The figures represent an endothermic pattern for the HRP–SDS interaction, whereas an exothermic trend is observed for the HRP–DTAB interaction. Among the mentioned enthalpies, ΔH_{unf} and ΔH_{bin} characterize the interaction of HRP with surfactants. ΔH_{unf} gives the extent of

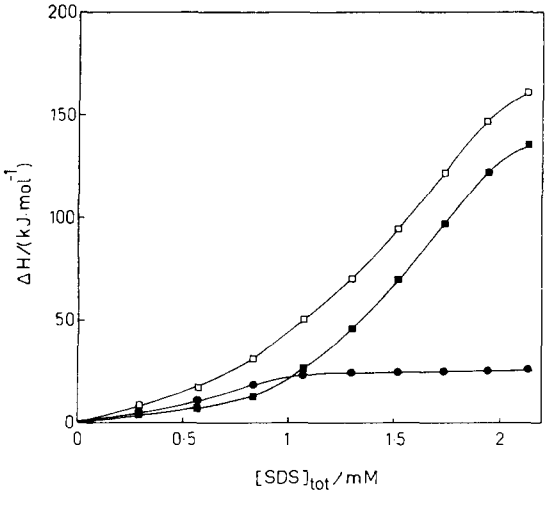

Fig. 3. Enthalpy changes on the interaction of HRP with SDS at pH 6.4 and 27°C. □ calorimetric; ■ binding and • unfolding.

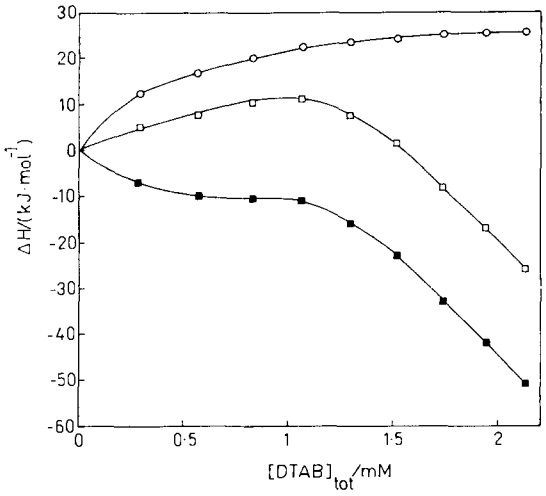

Fig. 4. Enthalpy changes on the interaction of HRP with DTAB at pH 6.4 and 27°C. □ calorimetric; ■ binding and ○ unfolding.

unfolding raised by electrostatic interactions at transition concentration for peroxidase. Fig. 5(a,b) indicates that unfolding of HRP becomes complete at detergent concentrations, $[S]_{1/2}$, corresponding to the g_1 values. In other words, the unfolding process depends on the ionic interactions, and the enthalpies of unfolding at g_1 points involve 95 and 97% of the related overall unfolding enthalpies for SDS and DTAB, respectively. This means that the hydrophobic interactions contribute poorly in unfolding of HRP by the surfactants, whereas urea and guanidine hydrochloride as chemical denaturants indicate a large contribution of hydrophobic interactions in the unfolding of HRP [10].

Fig. 5(a,b) also shows the dependence of $\Delta H_{bin}(\overline{\nu})$ on $\overline{\nu}$. $\Delta H_{bin}(\overline{\nu})$ is an approximate value of ΔH_{bin} due to binding of 1 mol of surfactant to 1 mol of HRP at the corresponding $\overline{\nu}$ value ($\Delta H_{bin}/\overline{\nu}$). The maxima and minima on the $\Delta H_{bin}(\overline{\nu})$ curves show the coincidence of g_1, $[S]_{1/2}$ and saturation point at ΔH_{unf} curve. This appearance means that the $[S]_{1/2}$ point is the position of the end of ionic interaction and start of hydrophobic contribution. Although the binding of surfactants continues at $\overline{\nu} > g_1$ but the unfolding process of HRP was mostly completed by both the anionic and cationic detergents. Thus, $\Delta H_{bin}(\overline{\nu})$ could be introduced as a subtle parameter for the clear indication of transition concentration ($[S]_{1/2}$) or the saturation point of ionic binding sites (g_1) for the interaction of HRP with ionic surfactants. It also

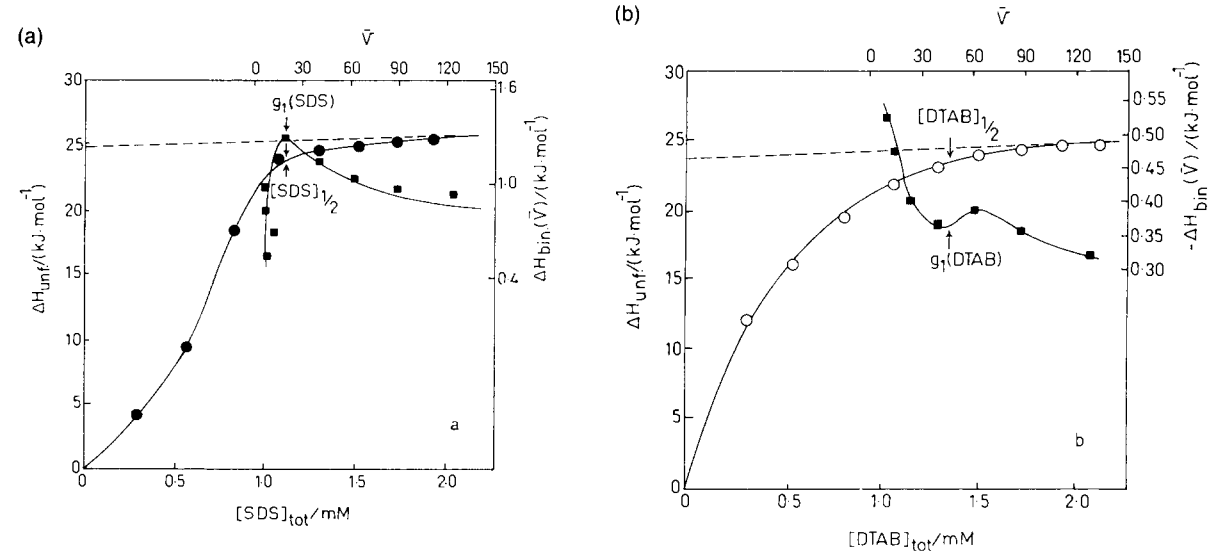

Fig. 5. (a) • Dependence of ΔH_{unf} on the total concentration of SDS; ■ plot of $\Delta H_{bin.}(\bar{\nu})$ vs. $\bar{\nu}$ (SDS) at pH 6.4 and 27°C; (b) ○ Dependence of ΔH_{unf} on the total concentration DTAB; ■ plot of $\Delta H_{bin.}(\bar{\nu})$ vs. $\bar{\nu}$ (DTAB) at pH 6.4 and 27°C.

separates the electrostatic (for $\bar{\nu} < g_1$) and the hydrophobic (for $\bar{\nu} > g_1$) portions from each other by this parameter.

Acknowledgements

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