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Enthalpy and Heat Capacity Changes for Formation of Compound I with Horseradish Peroxidase and of Complexes with Benzhydroxamic Acid and Benzhydrazide

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ABSTRACT: Calorimetric measurements were performed on the formation of compound I and of complexes of horseradish peroxidase with benzhydroxamic acid and benzhydrazide at pH 7.0 and at 9.4, 15, and 25 °C. The enthalpy and heat capacity changes for the formation of compound I are −111.2 kJ/mol and −1.75 kJ/(K mol) at 25 °C, respectively. By combining these results with other available data for related reactions, it can be shown that the heme-coordinated oxygen atom is energetically stabilized only about 10 kJ/mol compared to the oxygen atom in its molecular state. Thermodynamic quantities for the binding of benzhydroxamic acid to the enzyme were determined from calorimetric results and equilibrium titration data. At 25 °C, the values are $\Delta G°$ = −31.1 kJ/mol, $\Delta H°$ = −58.3 kJ/mol, $\Delta S°$ = −0.09 kJ/(K mol), and ΔC_p = 0.02 kJ/(K mol). The corresponding values for the binding of benzhydrazide to peroxidase are $\Delta G°$ = −21.7 kJ/mol, $\Delta H°$ = −42.4 kJ/mol, $\Delta S°$ = −0.07 kJ/(K mol), and ΔC_p = 0.94 kJ/(K mol) at 25 °C. It is evident that the most striking difference between the two processes is the large difference in their heat capacity changes.

Compound I is an active intermediate formed during reaction between native peroxidase and hydrogen peroxide or organic peracids:

$$E + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

where E is native peroxidase (Dunford & Stillman, 1976). Compound I formation reactions with hydrogen peroxide or aromatic peracids are very fast. The bimolecular rate constants are around diffusion controlled (Dunford & Hewson, 1977; Nakatani & Dunford, 1979). During reaction, an oxygen atom is trapped into the heme iron, which is then converted to ferryl from ferric, and the cation radical of hemin is produced (Fujita et al., 1983). Compound I reacts as an electron acceptor with second substrates (Dunford & Stillman, 1976). Although many spectroscopic studies of compound I have been reported, its formation has not as yet been thermodynamically characterized. Compound I is not a very stable intermediate, but its formation is practically irreversible. This enables the enthalpy change in the reaction to be determined by direct calorimetric measurement, although the Gibbs energy data cannot be readily determined.

Specific ligands such as aromatic hydroxamic acids and azides bind strongly to horseradish peroxidase (Shonbaum, 1973). Their dissociation constants are measurable by spec-

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Table I: Observed Dissociation Constants and Enthalpy Changes for Reaction of Peroxidase with Hydrogen Peroxide, Benzhydroxamic Acid, and Benzhydrazide at pH 7.0 in 0.01 M Phosphate Buffer Containing 0.1 M KNO_3 and at Various Temperatures[a]

ligand	K_d (25 °C) (μmol dm^{-3})	ΔH (kJ/mol) 9.4 °C	15.0 °C	25.0 °C
H_2O_2	*b*	−81.5 ± 6.6 (9)	−97.6 ± 2.4 (7)	−109.8 ± 3.2 (20)
benzhydroxamic acid	3.52	−60.2 ± 2.9 (7)	−56.2 ± 1.0 (7)	−59.2 ± 0.7 (19)
benzhydrazide	157	−56.5 ± 2.4 (7)	−52.5 ± 1.8 (7)	−42.1 1.5 (8)

[a] K_d is the dissociation constant determined from spectrophotometric titration at 25 °C. ΔH is the enthalpy change of reaction with respective ligand at indicated temperatures. The values of ΔH for the formation of complexes with benzhydroxamic acid and benzhydrazide are those corrected for the unbound fraction of peroxidase in the reaction mixture. The numbers in parentheses indicate the number of measurements. [b] Immeasurably small.

troscopic titration, and therefore, if calorimetric measurements are conducted, detailed thermodynamic information about the formation of these complexes will be produced. In this study, the enthalpy and heat capacity changes associated with the formation of compound I and of the complexes of benzhydroxamic acid and benzhydrazide with horseradish peroxidase were calorimetrically determined.

Materials and Methods

Horseradish peroxidase (EC 1.11.1.7) was purchased from Boehringer Manheim GmbH, Manheim, West Germany. The ratio of absorbances at 403 and 280 nm was greater than 3.0. The enzyme concentration was determined spectrophotometrically by using a specific absorption of 1.02×10^5 M^{-1} cm^{-1} at 403 nm (Schonbaum & Lo, 1972). Hydrogen peroxide was from Nakarai Chemicals Ltd., Kyoto, Japan: the concentration was determined by the absorbance at 240 nm (Nelson & Kiesow, 1972). Benzhydroxamic acid and benzhydrazide, both of reagent grade, were also purchased from Nakarai Chemicals Ltd. They were used without further purification. The measurements were made in 0.01 M phosphate buffer, pH 7.0, containing 0.1 M KNO_3.

The dissociation constants for ligand binding were determined by spectrophotometric titration in a Union Giken SM-401 spectrophotometer. Calorimetric measurements were performed in a flow calorimeter with a gold-tubing cell having a volume of 0.90 cm^3. Details of the calorimeter and of its mode of operation have been published previously (Takahashi & Fukada, 1985). The measurements were made with the stopped-flow procedure, each solution being delivered at a flow rate of 0.137 cm^3/min for 120–180 s. The flow rate was frequently checked by weighing the effluent. The calorimeter was calibrated at each temperature by measuring the heat of neutralization of 0.004 mol dm^{-3} HCl by 0.01 mol dm^{-3} NaOH with the values reported by Grenthe et al. (1970). To obtain the net enthalpy of reaction, the heats of dilution of the reactants and the viscous heating due to mixing buffer with buffer, which were usually very small, were substracted from the total heat change observed. The concentration of peroxidase was usually about 20 μmol dm^{-3} after mixing. The concentration of hydrogen peroxide was slightly less than that of enzyme, so that it was all reacted with the peroxidase. The concentrations of benzhydroxamic acid and of benzhydrazide were in the order of 3.5 mmol dm^{-3} and 15 mmol dm^{-3}, respectively, both after mixing. Corrections were applied for the unbound fraction of the peroxidase on the basis of the equilibrium data determined separately.

Results and Discussion

Compound I Formation. Compound I formation from the peroxidase and hydrogen peroxide is characterized by a large heat evolution. Figure 1 shows a calorimetric titration with hydrogen peroxide at 25 °C. The measurements were conducted with excess peroxidase to prevent any secondary reactions that may take place with hydrogen peroxide present in excess. Since it has been shown (Yamada & Yamazaki, 1974) that neither uptake nor release of protons takes place during the reaction, no correction for the heat of buffer protonation was necessary. The enthalpy change for the formation of compound I was determined from the slope of the above titration. The results obtained at 9.5, 15, and 25 °C are summarized in the third, fourth, and fifth columns of Table I. The enthalpy change varied linearly with the temperature of measurement, and the heat capacity change was evaluated as the temperature coefficient of the enthalpy, the least-squared results being $\Delta H = -111.2 \pm 4.5$ kJ/mol at 25 °C and $\Delta C_p = -1.75 \pm 0.42$ kJ/(K mol). The enthalpy change for the decomposition of hydrogen peroxide into oxygen and water in aqueous solution is reported by Nelson and Kiesow (1972) to be

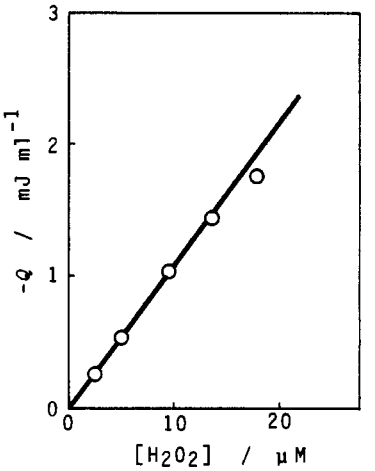

FIGURE 1: Heat of compound I formation from horseradish peroxidase and hydrogen peroxide observed at pH 7.0 in 0.01 M phosphate buffer containing 0.1 M KNO_3 at 25 °C. The peroxidase solution of 36.2 μM was mixed with hydrogen peroxide solutions in the gold-tubing cell of a flow calorimeter. The solutions were sent through two Teflon inlet tubings by a syringe-drive pump as described previously (Takahashi & Fukada, 1985). The amounts of heat change observed were plotted against hydrogen peroxide concentration. From the slope of the plot, the enthalpy change of compound I formation was calculated to be $\Delta H = -109.8 \pm 3.2$ kJ/mol.

$$H_2O_2 \rightarrow H_2O + {}^1/_2O_2$$
$$\Delta H = -100.8 \text{ kJ/mol} \quad (2)$$

From this result and our value for the formation of compound I, we have for the hypothetical thermochemical reaction

$$E + {}^1/_2O_2 \rightarrow \text{compound I}$$
$$\Delta H = -10.4 \text{ kJ/mol} \quad (3)$$

Thus, heme-coordinated oxygen atom is energetically stabilized only about 10 kJ/mol compared to the oxygen atom in its molecular state. This relatively small extent in the energetic stabilization of oxygen atom in compound I seems to reflect

the trapping capacity of active oxygen in enzyme peroxidase that is further utilized in oxidizing the second substrate with a high efficiency. Although the comparison should essentially be made on the Gibbs energy change, it is our view that even the energy term alone characterizes the thermodynamic properties of compound I as an active intermediate.

The enthalpy change for the reaction between compound I and the third materials (the second substrates) can be evaluated by combination with appropriate data that are available in literatures. For example, from eq 1–3, the enthalpy change of decomposition of hydrogen peroxide by compound I is given as

$$\text{compound I} + H_2O_2 \rightarrow E + H_2O + O_2$$
$$\Delta H = -90.4 \text{ kJ/mol} \quad (4)$$

The net reaction of eq 1 and 4 is substantially a peroxidase-catalyzed catalase reaction. On the other hand, the enthalpy of formation of water is −285.8 kJ/mol at 25 °C (Wageman et al., 1968). Combining this value with the enthalpy change in eq 3, we have for the enthalpy change of the hypothetical reaction between compound I and hydrogen molecule at 25 °C

$$\text{compound I} + H_2 \rightarrow E + H_2O$$
$$\Delta H = -275.4 \text{ kJ/mol} \quad (5)$$

Oxidation of ferrocyanide by protons to produce ferricyanide and hydrogen is accompanied by an enthalpy change of 223.4 kJ/mol (Hanania et al., 1967):

$$2Fe(CN)_6^{4-} + 2H^+ \rightarrow 2Fe(CN)_6^{3-} + H_2$$
$$\Delta H = 223.4 \text{ kJ/mol} \quad (6)$$

From eq 5 and 6, we find the enthalpy change for the oxidation of ferrocyanide by compound I to be

$$\text{compound I} + 2H^+ + 2Fe(CN)_6^{4-} \rightarrow E + H_2O + 2Fe(CN)_6^{3-}$$
$$\Delta H = -52.0 \text{ kJ/mol} \quad (7)$$

The above stoichiometry concerning proton balance has been proved potentiometrically (Yamada & Yamazaki, 1974). From eq 1 and 7, the overall enthalpy change for reaction between hydrogen peroxide and ferrocyanide is

$$H_2O_2 + 2H^+ + 2Fe(CN)_6^{4-} \rightarrow 2H_2O + 2Fe(CN)_6^{3-}$$
$$\Delta H = -163.2 \text{ kJ/mol} \quad (8)$$

This result indicates that about two-thirds of the total enthalpy change of peroxidase-catalyzed ferrocyanide oxidation is ascribable to the formation of compound I (−111.2 kJ/mol). Actually, compound I is converted to compound II by ferrocyanide, and the native enzyme is recovered in a subsequent reaction as indicated by the following stoichiometric equations (Yamada & Yamazaki, 1974):

$$\text{compound I} + H^+ + Fe(CN)_6^{4-} \rightarrow \text{compound II} + Fe(CN)_6^{3-} \quad (9)$$

$$\text{compound II} + H^+ + Fe(CN)_6^{4-} \rightarrow E + Fe(CN)_6^{3-} + H_2O \quad (10)$$

The reaction in eq 10 is known to be the rate-determining step of the overall enzyme reaction (Cotton & Dunford, 1973). However, the enthalpy changes for the above two processes are not known at the present time, although we can state that the sum of enthalpy contributions due to eq 9 and 10 amounts to −52 kJ/mol.

Table II: Thermodynamic Quantities for Formation of Compound I and of Complexes with Benzhydroxamic Acid and Benzhydrazide at pH 7.0 and 0.01 M Phosphate Buffer Containing 0.1 M KNO_3 and at 25 °C[a]

ligand	$\Delta G°$ (kJ/mol)	ΔH (kJ/mol)	$\Delta S°$ [kJ/(K mol)]	ΔC_p [kJ/(K mol)]
H_2O_2		−111.2 ± 4.5		−1.75 ± 0.42
benzhydroxamic acid	−31.1	−58.3 ± 2.8	−0.09	0.02 ± 0.26
benzhydrazide	−21.7	−42.4 ± 0.9	−0.07	0.94 ± 0.08

[a] All quantities were derived from the experimental data by the method of regression analysis on the basis of eq 11–14.

Formation of Complexes with Ligands. Dissociation constants of complexes of peroxidase with benzhydroxamic acid and benzhydrazide were determined by spectrophotometric titration and are given in the second column of Table I. Enthalpy changes determined calorimetrically at three different temperatures are also shown in the table. Since the dissociation constants are independent of pH in the neutral pH range (Schonbaum, 1973), we assumed that there is neither uptake nor release of protons during the ligand binding (Laskowski & Finkenstadt, 1972), and therefore, no buffer corrections were made.

The enthalpy changes for the binding of the two ligands obtained at three different temperatures are summarized in the third, fourth, and last columns of Table I. A linear regression analysis was made of these data on the basis of

$$\Delta H = \Delta H(298) + \Delta C_p(T - 298.15) \quad (11)$$

and the enthalpy change at 25 °C [$\Delta H(298)$] and the heat capacity change (ΔC_p) were determined. By combining these values with the standard Gibbs energy changes of reaction, which were derived from the dissociation constant by

$$\Delta G°(298) = (-R \times 298.15) \ln (1/K_d) \quad (12)$$

the thermodynamic quantities were calculated as a function of temperature according to eq 11 and the following equations:

$$\Delta G° = [\Delta H(298) - 298.15\Delta C_p](1/T - 1/298.15)T + T\Delta C_p \ln (T/298.15) + \Delta G°(298) \quad (13)$$

$$\Delta S° = \Delta S°(298) + \Delta C_p \ln(T/298.15) \quad (14)$$

The values at 25 °C are summarized in Table II together with the data for the formation of compound I. The temperature variations of the thermodynamic parameters are illustrated in Figure 2. The open and closed circles are the enthalpy changes that were determined calorimetrically for benzhydroxamic acid and benzhydrazide, respectively. From Table II and Figure 2, it is evident that there is a remarkable difference in the heat capacity change for the two binding processes; while ΔC_p for the binding of benzhydroxamic acid is nearly 0, the binding of benzhydrazide is accompanied by a relatively large increase in heat capacity. Both of the processes are of the same nature in the sense that they are characterized by a favorable enthalpy and small unfavorable entropy changes. However, because of the large difference in ΔC_p, the contributions of the enthalpy and the entropy may be quite different for the two ligand bindings depending on the temperature. The binding of benzhydroxamic acid is entropically more unfavorable than that of benzhydrazide at above 18 °C and it is energetically more favorable than that of the latter at above 9 °C.

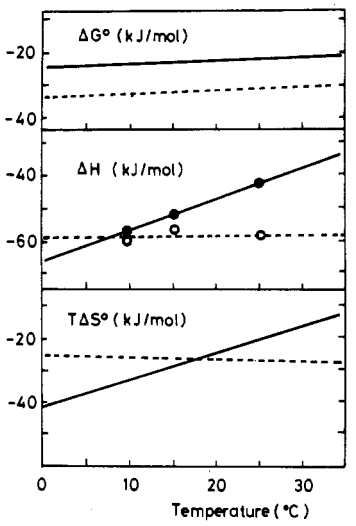

FIGURE 2: Temperature variations of the thermodynamic quantities for the complex formation of peroxidase with benzhydroxamic acid (--) and benzhydrazide (—) at pH 7.0 in 0.01 M phosphate buffer containing 0.1 M KNO_3. The circles are the calorimetrically determined values.

According to Schonbaum (1973), the polar moieties of the two ligands contain an intramolecular hydrogen bond to form five-membered rings of about the same size at neutral pH:

It is surprising that, in spite of this structural similarity, the binding processes of the two ligands are thermodynamically quite different. Sturtevant (1977) proposed a method for interpreting entropy and heat capacity data in terms of contributions made by changes in hydrophobic interactions and vibrational modes. Applying this method to the present data, we find that in the binding of benzhydroxamic acid the contribution of the changes in vibrational modes is larger than that of the changes in hydrophobic interactions, while the latter is predominant in the binding of benzhydrazide.

Both of these processes are regarded as involving multidentate binding to the peroxidase, with hydrophobic interactions between benzene rings of the ligands and the substrate binding site, and coordination or hydrogen bonding between the polar group of the ligands and heme iron or heme-coordinated water molecules (Schonbaum, 1973; Morishima & Ogawa, 1979). It is possible that the binding modes of these multidentate ligands to the enzyme may be flexible, depending on the geometrical fitness and strength of the interactions, and that this situation may result in the differences in the thermodynamic properties observed in this study.

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Registry No. H_2O_2, 7722-84-1; peroxidase, 9003-99-0; benzhydroxamic acid, 495-18-1; benzhydrazide, 613-94-5.

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