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HIGH-PRESSURE EFFECT ON THE EQUILIBRIUM AND KINETICS OF CYANIDE BINDING TO CHLOROPEROXIDASE

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The kinetics of cyanide binding to chloroperoxidase were studied using a high-pressure stopped-flow technique at 25°C and pH 4.7 in a pressure range from 1 to 1000 bar. The activation volume change for the association reaction is $\Delta V^{\ddagger} = -2.5 \pm 0.5$ ml/mol. The total reaction volume change, determined from the pressure dependence of the equilibrium constant, is $\Delta V^{o} = -17.8 \pm 1.3$ ml/mol. The effect of temperature was studied at 1 bar yielding $\Delta H^{\ddagger} = 29 \pm 1$ kJ/mol, $\Delta S^{\ddagger} = -58 \pm 4$ J/mol per K. Equilibrium studies give $\Delta H^{o} = -41 \pm 3$ kJ/mol and $\Delta S^{o} = -59 \pm 10$ J/mol per K. Possible contributions to the binding process are discussed: changes in spin state, bond formation and conformation changes in the protein. An activation volume analog of the Hammond postulate is considered.

1. Introduction

Activation volumes are frequently used in organic and inorganic chemistry as a criterion for the mechanism of reactions [1]. Studies on biological processes are beginning to reveal interesting insight into the mechanism of ligand binding to proteins [2–4]. A number of high-pressure studies of heme proteins have been published, reporting data on the effect of pressure on denaturation of the protein [5], spectroscopic properties [6–8], spin-state equilibria [9,10] and ligand binding [11–13].

When high hydrostatic pressure is applied to heme proteins two main equilibrium effects are observed. The primary effect observed for proteins that are in a thermal high spin-low spin equilibrium is a shift towards the low-spin form [9,10]. For proteins that have a large high-spin content the applied pressure has to be large before this effect is observed, more than 8000 bar in the case of horseradish peroxidase [9]. For methemoglobin, metmyoglobin and their azide and fluoride complexes the optical spectrum of the pressure-induced low-spin species suggests that it is a closed crevice structure wherein the heme-linked ligand at the sixth coordination position is replaced by the distal histidine. The presence of high-affinity ligands (like cyanide) stabilizes the open crevice structure [9]. For complexes that are already in a low-spin state only small subtle structural changes in the heme environment could be observed [10]. The second effect which may occur at higher pressures (> 2000–8000 bar, depending upon the enzyme) is very similar to denaturation by heat, acid or temperature [5].

We have investigated and report here the effect of pressure and temperature on the equilibrium and kinetics of cyanide binding to chloroperoxidase isolated from *Caldariomyces fumago*. It is the first time a high-pressure study has been undertaken for this enzyme.

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2. Experimental section

Chloroperoxidase (chloride:hydrogen-peroxide oxidoreductase. EC 1.11.1.10) was purified from *C. fumago* using the previously described methodology [14] after growth in a defined fructose-salt medium [15]. The RZ value (A_{400}/A_{280}) of the sample was 1.38–1.40 for the pressure experiments. A different enzyme sample with $RZ = 1.39$ was used for the temperature experiments. Chloroperoxidase concentrations were determined spectrophotometrically using an extinction coefficient ϵ_{400} of 87.4 mM^{-1} cm^{-1} at pH 5 [16]. Potassium cyanide was reagent grade and used without further purification. The cyanide content of the salt was determined by titration with a standardized silver nitrate solution using diphenylcarbazide as indicator [17]. The value obtained was used to calculate concentrations by weight. An unbuffered cyanide stock solution (0.1 M) was prepared within 4 h of all experiments and the required amount of potassium cyanide was transferred from the stock solution to a buffered solution immediately before every experiment. The buffer used in all the experiments was a citric acid-sodium hydroxide buffer with ionic strength 0.01 and pH 4.6 or 4.7 measured at room temperature and 1 bar. The final ionic strength was adjusted to 0.11 using potassium sulfate as an inert salt. Changes in pH due to the temperature or pressure dependence of the ionizations of citric acid [18] (up to 0.6 pH units towards the acid region in the pressure range of the study) were neglected because the equilibrium and rate constants under investigation are not dependent upon the H^+ concentration at 1 bar in the region of pH 4.7 [19]. All buffer components and salts were reagent grade.

Light absorption measurements at 1 bar were performed on a Cary 118 spectrophotometer. For absorption measurements at high pressure a single-beam Zeiss PM QII spectrophotometer was used. The instrument was rebuilt to adapt it to high-pressure measurements. A 11 μM chloroperoxidase solution was incubated with 0.14 and 0.18 mM potassium cyanide in two separate sets of experiments. From the change in absorbance measured at the chloroperoxidase-cyanide peak at 437 nm a value for the equilibrium constant (K) is obtained. The extinction coefficients at 437 nm used in the calculations were 30.3 mM^{-1} cm^{-1} for native chloroperoxidase and 80.3 mM^{-1} cm^{-1} for its cyanide complex. The pressure was raised in 200-bar increments from 1 to 1400 bar and the solution was allowed to equilibrate (3 or 4 min) before the absorption value was read. Due to compression of the solution the observed absorbance is too high. To compensate for this effect the observed absorbances are multiplied by the relative volume of water (volume at pressure P divided by volume at 1 bar) prior to calculation of K at each pressure. The values for the relative volume of water are taken from ref. 20. At the end of the experiment the pressure was released slowly in one or two steps and after equilibrium at 50 bar (usually 15 min) the absorption value always returned to the original value within 0.002 absorption units. The procedure was repeated twice more with the same solution and the mean value at every pressure was used in the calculations.

High-pressure kinetic experiments were performed on a stopped-flow apparatus specifically designed for this purpose [21]. The dead time of the instrument is approx. 20 ms.

Kinetic experiments to obtain rate constants at 1 bar as a function of the temperature were performed in a Union Giken rapid reaction analyzer Model RA601 in the stopped-flow mode. The chloroperoxidase concentration was 1 μM. The cyanide concentration was varied with 0.25 mM increments between 0.25 and 2 mM. Six traces were recorded for every cyanide concentration at a given temperature and the mean value for the observed rate constant was used in the calculations.

The equilibrium constant was determined by adding microliter amounts of a potassium cyanide solution (0.05, 0.1 or 0.2 M) to 2 ml of chloroperoxidase solution (5.2 ± 0.1 μM). The final volume added never exceeded 0.02 ml. After every addition the absorbance at 437 nm was recorded with a Cary 219 spectrophotometer. For both kinetic and equilibrium measurements the temperature was varied between 5 and 35°C with increments of 5 ± 0.3°C. Linear plots were fitted using a linear least-squares program with $1/\sigma^2$ as weighting factor where σ is the standard deviation. Stopped-flow

traces were computer fitted as exponentials using a nonlinear least-squares program.

3. Results

At pH 4.7 virtually all of the cyanide in solution exists as hydrocyanic acid ($pK_A = 9.2$) [22]. The chloroperoxidase(ClP)-cyanide complexation can be written as follows

$$ClP + HCN \underset{k_-}{\overset{k_+}{\rightleftharpoons}} ClP-CN \quad (1)$$

Under pseudo first-order conditions the observed rate constant k_{obs} is given by the following equation

$$k_{obs} = k_+ [KCN]_o + k_- \quad (2)$$

where $[KCN]_o$ is the concentration of potassium cyanide added (in large excess over that of chloroperoxidase) and k_+ and k_- the second-order association rate constant and the first-order dissociation rate constant for the reaction. Plots of k_{obs} vs.

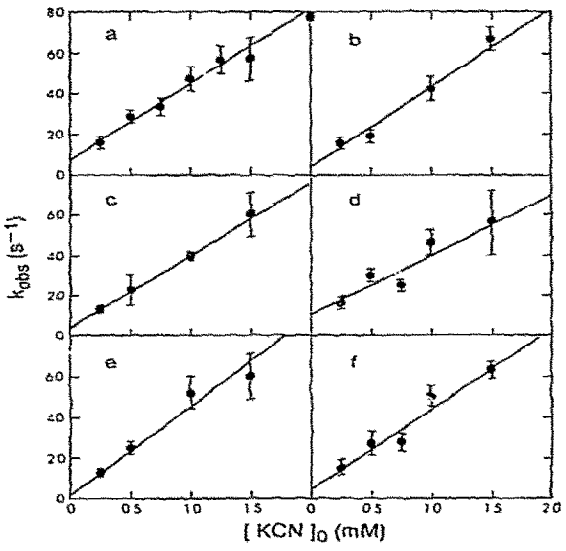

Fig. 1. Dependence of the pseudo-first-order rate constant (k_{obs}) on the excess of potassium cyanide at 1 (a), 200 (b), 400 (c), 600 (d), 800 (e) and 1000 bar (f). The chloroperoxidase concentration is 2 μM.

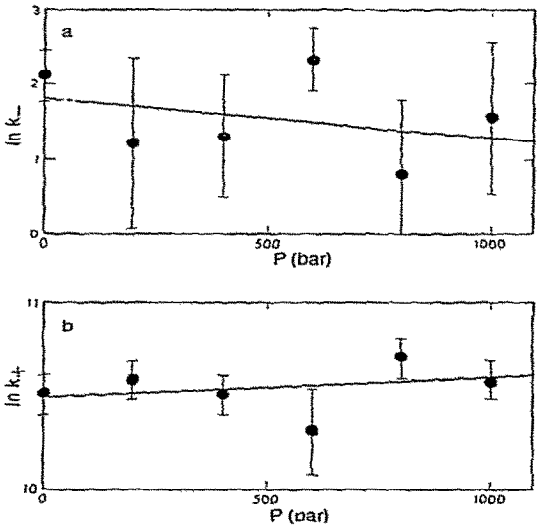

Fig. 2. Dependence of $\ln k_-$ (top) and $\ln k_+$ (bottom) on pressure. The values for k_- and k_+ are obtained from the intercepts and slopes of the plots in fig. 1.

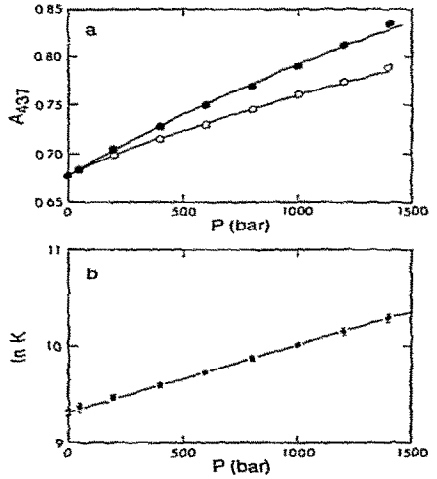

Fig. 3. Pressure dependence of the spectra and of the cyanide association equilibrium constant, K (M^{-1}). (a) The closed circles represent the observed absorbance of the cyanide-chloroperoxidase complex (437 nm) and the open circles represent the absorbance values corrected for the compressibility of water. (b) The values of K calculated from the corrected absorbance changes at 437 nm. The reaction volume change is obtained from the slope which is $-\Delta V^\circ/RT$.

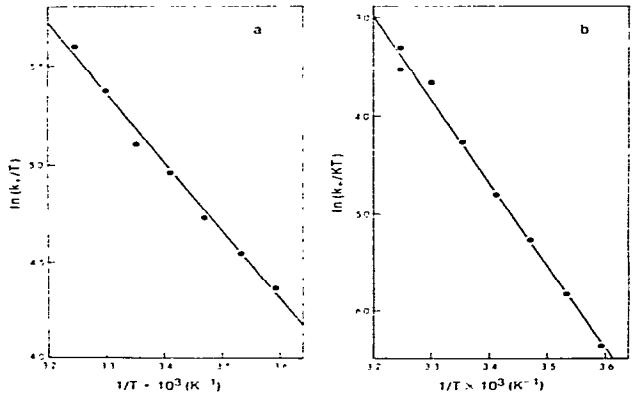

Fig. 4. (a) Plot of $\ln(k_+/T)$ vs. $1/T$. The slope is equal to $-\Delta H_+^{\ddagger}/R$ for the association reaction. (b) Plot of $\ln(k_-/KT)$ vs. $1/T$, where $k_+/K = k_-$. The slope yields $-\Delta H_-^{\ddagger}/R$ for the dissociation reaction. The value of $\Delta H_-^{\ddagger}$ obtained in this manner is more accurate than that obtained directly from the dissociation rate constants.

potassium cyanide concentration were used to obtain values for k_+ and k_- as a function of the pressure at 25°C (fig. 1) and as a function of the temperature at 1 bar (plots not shown). The pressure dependence of k_+ and k_- is shown in fig. 2. The values of k_- obtained from plots such as in fig. 1 are subject to large experimental errors due to the extrapolation from relatively large numbers to an intercept near the origin. The large error in $\Delta V_-^{\ddagger}$ obtained from the slope of the secondary plot shown in fig. 2 is a reflection of the error in k_-. However, a more accurate value for $\Delta V_-^{\ddagger}$ can be obtained using the effect of pressure on the equilibrium. Because of the logarithmic relationship that exists between reaction volume change (ΔV°) and equilibrium constant (K) it follows that from $K = k_+/k_-$ one obtains $\Delta V^{\circ} = \Delta V_+^{\ddagger} - \Delta V_-^{\ddagger}$. The equilibrium constant was determined in the high-pressure spectrophotometer as described in section 2. A plot of ln K vs. pressure allows us to calculate the change in reaction volume (fig. 3). The mean value for ΔV° obtained from two experiments is -17.8 ± 1.3 ml mol^{-1}. The activation volume changes obtained from fig. 2 are $\Delta V_+^{\ddagger} = -2.5 \pm 0.5$ ml mol^{-1} for the forward reaction and $\Delta V_-^{\ddagger} = 12 \pm 17$ ml mol^{-1} for the dissociation reaction. Using the experimental value for ΔV° and $\Delta V_+^{\ddagger}$ obtained from the kinetic experiments, $\Delta V_-^{\ddagger} = \Delta V_+^{\ddagger} - \Delta V^{\circ} = -2.5 - (-17.8) = 15.3$ ml mol^{-1} with a cumulative error of $0.5 + 1.3 = \pm 1.8$ ml mol^{-1}.

The effect of temperature on the rate constants at 1 bar is shown in fig. 4. The activation parameters were calculated according to eq. 3

$$\ln\frac{k}{T} = \ln\frac{\bar{k}}{h} + \frac{\Delta S^{\ddagger}}{R} - \frac{\Delta H^{\ddagger}}{RT} \tag{3}$$

where T is the temperature (in K), $\bar{k}$ the Boltzmann constant, h Planck's constant, R the universal gas constant and $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ the entropy and enthalpy of activation. Again it proved more accurate to use the k_- calculated from k_+ and the equilibrium constant instead of the extrapolated k_- value. The equilibrium constant was obtained by plotting the observed absorbance change at 437 nm relative to the added cyanide concentration according to the Scatchard equation

$$\frac{(A - A_o)}{[\mathrm{ClP}]_o}\frac{1}{[\mathrm{KCN}]} = \Delta\epsilon K - K\frac{(A - A_o)}{[\mathrm{ClP}]_o} \tag{4}$$

where A_o and A are the absorbance before and after the addition of cyanide, $[\mathrm{ClP}]_o$ the total enzyme concentration, $\Delta\epsilon$ the change in extinction coefficient at 437 nm due to total complexation and K the association equilibrium constant. The thermodynamic parameters were obtained from a plot of ln K vs. $1/T$, $\Delta H^{\circ} = -41 \pm 3$ kJ mol^{-1} and $\Delta S^{\circ} = -59 \pm 10$ J mol^{-1} K^{-1}. The activation parameters obtained from fig. 4 are $\Delta H_+^{\ddagger} = 29 \pm 1$ kJ mol^{-1}, $\Delta S^{\ddagger +} = -58 \pm 4$ J mol^{-1} K^{-1}, $\Delta H_-^{\ddagger} = 70 \pm 3$ kJ mol^{-1} and $\Delta S_-^{\ddagger} = 3 \pm 10$ J mol^{-1} K^{-1}.

4. Discussion

At pH 4.7 and room temperature chloroperoxidase is mostly in the high-spin state [16]. At 1 bar when the pH is raised above pH 7-chloroperoxidase undergoes an irreversible transition to a species with unique optical properties that is probably a closed-crevice configuration of the heme [23]. The fact that changes in absorbance under pressure were completely reversible upon release of the pressure indicates that none of this species

is formed under the conditions used for these experiments. As with horseradish peroxidase [13] no denaturation of chloroperoxidase was observed in the pressure range used in this study.

The volume change for cyanide binding to chloroperoxidase may be the sum of several effects: contraction due to the high spin-low spin transition upon cyanide binding, shortening of the iron-ligand bonds upon cyanide binding, conformational changes in the protein, possible charge neutralization or separation during the reaction. It is possible to evaluate some of these effects from our knowledge of the active site of chloroperoxidase. From extended X-ray absorption-fine structure studies [24] it is known that upon the transition from high-spin to low-spin chloroperoxidase the iron moves from an out-of-plane position into the plane of the porphyrin and simultaneously there is a contraction of the iron-pyrrole nitrogen distance of 0.05 Å, the iron–α-carbon (of the porphyrin) distance of 0.02 Å and the iron-sulfur (fifth ligand) bond of approx. 0.11 Å. There is also a contraction of the radius of the iron atom by approx. 0.12 Å. However, we agree with the assessment that these combined effects could only be of the order of 0.2–0.3 ml/mol heme [12].

For cyanide binding to peroxidases it is believed that only the electrically neutral ligand reacts [25]. However, hydrocyanic acid must lose a proton before bond formation with the iron. So the total volume change must contain the ionization volume of hydrocyanic acid, $\Delta V_{HCN} = -6.5$ ml/mol [12]. If the proton is trapped by a neutral basic group the volume change for this step is expected to be small, since the net charge has not changed. The volume change for a proton added to a carboxyl group is 10–12 ml/mol [18]. If the proton released from hydrocyanic acid is trapped by the buffer the volume change is given by the negative value of the ionization volume for the buffer (12.7 ml/mol for the second ionization of citric acid [18]). The fact that the activation volume change ($\Delta V_+^\ddagger$) for the binding is small can be explained by the compensating effect of cyanide dissociation and protonation of a carboxyl group (either on the protein or the buffer). The presence of a heme-linked acid group with $pK_A < 3$ that has to be deprotonated to allow cyanide to bind has been demonstrated [19]. The explanation of proton transfer to carboxylate was offered to account for the $\Delta V_+^\ddagger$ observed in cyanide binding to horseradish peroxidase. The total volume change was not determined [13]. The activation parameters for cyanide binding to horseradish peroxidase are $\Delta V_+^\ddagger = 1.7 \pm 0.5$ ml mol^{-1} and by application of eq. 3 to the data in ref. 13, $\Delta H_+^\ddagger = 27.4 \pm 0.7$ kJ mol^{-1} and $\Delta S_+^\ddagger = -57 \pm 2$ J mol^{-1} K^{-1}. With the exception of the change in sign for the small value of $\Delta V_+^\ddagger$, the results for cyanide binding to horseradish and chloroperoxidase are identical within experimental error [13].

Since a 2.5 ml/mol decrease in volume occurs upon formation of the transition state there is a further 15.4 ml/mol decrease to be accounted for upon formation of the low-spin cyanide complex. For cyanide binding to methemoglobin the reaction volume is -17.5 ml/mol at pH 6.5 of which approx. 10 ml/mol was attributed to protein conformation changes [12]. A change in conformation of the protein may not be a necessary assumption, since relatively large volume changes have been reported for spin-state transitions: the conversion from high spin to low spin occurred with $\Delta V^\circ = -6.7$ ml/mol for the R conformation of methemoglobin and $\Delta V^\circ = -12.2$ and -12.5 ml/mol for methemoglobin T and metmyoglobin, respectively [28]. Fe(III)-*N*,*N*′-dialkyldithiocarbamates show a 5–6 ml/mol expansion upon conversion from low to high spin [29]; changes of 10–20 ml/mol are observed for Fe(II) complexes with large ligands [30,31]. The spin-state transition upon cyanide binding may also have a contribution in the ΔH° and ΔS°, since the low-spin state is favored at low temperature for chloroperoxidase [16].

The kinetic and thermodynamic parameters obtained in this study for the binding of cyanide to chloroperoxidase are summarized in fig. 5 by means of reaction coordinate diagrams.

The observation that the activation volume constitutes only a small fraction of the reaction volume could be interpreted according to an activation volume analogy of the Hammond postulate [26]. The application of the Hammond principle to reactions under pressure has been proposed for organic reactions [27]. However, interpretation of

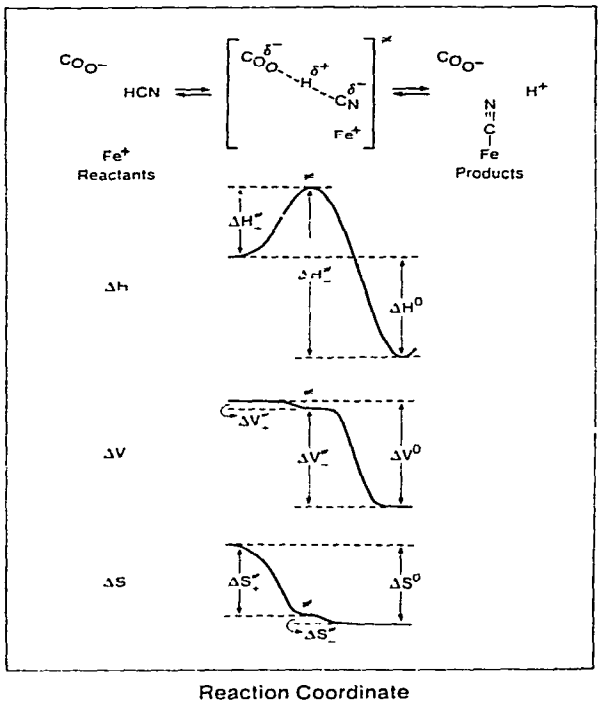

Fig. 5. Reaction coordinate diagrams for the binding of hydrogen cyanide to chloroperoxidase showing schematically the changes in ΔH, ΔV and ΔS for the forward and reverse reactions and the chemical equilibrium.

$\Delta V^{\ddagger}$ values may lead to different conclusions from those obtained from $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values [27]. In the absence of electrostatic interactions, activation volumes reflect changes in nuclear positions, while activation energies reflect the energetics involved in the flow of electrons during the disruption and/or formation of chemical bonds. Inspection of all the activation parameters for cyanide binding to chloroperoxidase suggests (allowing some fluctuation in volume due to charge delocalization in the transition state) that in terms of volume the transition state resembles the reactants while in terms of activation enthalpy the disruption of the H–CN bond is already in progress (fig. 5). Although there seems to be a correlation between the $\Delta V^{\ddagger}$ and $\Delta S^{\ddagger}$ values in terms of sign and magnitude, one occurs 'early' and the other 'late' along the reaction coordinate (fig. 5). Initial binding of cyanide is assisted by the interaction of hydrocyanic acid with an acid group in the vicinity of the active site, resulting in a small contraction upon formation of the transition state. The net volume change associated with ionization of hydrocyanic acid is small because it is compensated by the neutralization of an acid group on the protein or the buffer. The major volume change occurs upon formation of the products and is assigned to the high spin-low spin transition of the heme iron. Since only a small volume change can be calculated from the known contraction of the iron and the ligand bonds upon spin-state transition, the large observed volume change must be associated with long-range effects occurring in the protein and the solvent.

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