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Thermodynamics of the two step formation of horseradish peroxidase compound I

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Abstract. The effects of temperature (20 to −38 °C), pressure (normal pressures to 1.2 kbar) and solvent (water, 60% DMSO and 50% methanol) on the reaction of hydrogen peroxide or ethyl peroxide with horseradish peroxidase were studied. The formation of compound I was followed at 403 nm in a stopped flow apparatus adapted for high pressure and low temperature work.

As with the alkaline form (Job and Dunford 1978), the neutral form of the peroxidase binds peroxide substrates in two steps. It was the combined use of organic solvents and low temperatures which revealed saturation kinetics:

$$E + S \overset{K_1}{\rightleftharpoons} E\,S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{compound I},$$

where E = horseradish peroxidase and S peroxide substrate. In water and organic solvents at temperatures above −10 °C, K_1 was too small and k_2 too large to be measured, here $K_1 \cdot k_2$ was obtained. k_{-2} was too small for measurement under all conditions. Whereas K_1 was insentitive to the peroxide substrate and solvent composition, k_2 was very sensitive. The thermodynamic parameters $\Delta H^{\neq}$, $\Delta S^{\neq}$ and $\Delta V^{\neq}$ for K_1 and k_2 were obtained under different experimental conditions and the data are interpreted within the available thermodynamic theories.

Key words: Horseradish peroxidase compound I, solvent effect, hydrostatic pressure, subzero temperatures, stopped-flow

1. Introduction

Initially, cryoenzymology was the study of enzyme reactions in the presence of organic solvents as antifreezes. It was a means for slowing down these reactions so as to increase the temporal resolution and to stabilize intermediates for further study (Douzou et al. 1970; Douzou 1977 and references therein). More recently it became apparent that the usefulness of cryoenzymology could be extended.

- First, cryoenzymology requires the addition of an antifreeze (usually an organic solvent) to the medium and we have shown that by the use of this single perturbant one can obtain mechanistic information about an enzyme reaction pathway (Barman et al. 1983; Douzou et al. 1985; Barman et al. 1986). In addition, the action of the solvent on its own can lead to conclusions about the influence of the environment on enzyme activity, but this subject lacks a solid theoretical base (e.g. Biosca et al. 1983, 1984).
- Second, by changing the temperature, selected intermediates can be made to accumulate due to the perturbation of the associated equilibrium constants (Biosca et al. 1984).
- Finally, low temperatures combined with high pressures (cryobaroenzymology) enabled us to study the energetic features of the reaction leading to the formation of a transient state analogue complex of creatine kinase (Balny et al. 1985). In particular, we obtained the thermodynamic quantities associated with certain of the kinetic constants describing the formation of the complex.

The effect of temperature and pressure on a single step of an enzyme reaction pathway allows one to calculate the thermodynamic parameters $\Delta H^{\neq}$, $\Delta S^{\neq}$ and $\Delta V^{\neq}$ pertaining to the step. For the data to be fully informative the nature of the step must be known and for precision large temperature and pressure ranges must be used. But once again, because of the great complexities of the systems studied, there is a lack of a theoretical basis as to the significance of these thermodynamic quantities. However, by collecting as many quantities as possible and, in particular, by observing their dependence on the environment (solvent, pH, ionic strength etc.), one might

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eventually be able to come to conclusions as to the general concepts concerning the dynamics of the processes under study. This is one of the few avenues available for approaching the problem of protein dynamics in solution. It was from this angle that we initiated a study on the formation of the complex between horseradish peroxidase and its peroxide substrate (Compound I, Chance 1943; Kato et al. 1984; Paul et al. 1979).

Our aim, therefore, was to obtain the thermodynamic parameters of a single step on the horseradish peroxidase reaction pathway. Now, it is known that the alkaline form of the enzyme binds peroxides in two steps – i.e. that an ES type of complex precedes formation of compound I (Job and Dunford 1978). A two step binding process has not been demonstrated with the neutral form of the enzyme (e.g. Paul et al. 1979).

Our studies were carried out at neural pH by the use of stopped flow apparatuses, adapted to low temperature and high pressure work, constructed in this laboratory and by this means we were able to demonstrate a two-step process for the formation of compound I. The kinetics of the formation of compound I were followed using two peroxides (hydrogen peroxide and ethyl peroxide) with water, 60% DMSO or 50% methanol as solvent, in the temperature range +30 to −38 °C and at pressures up to 1.2 kbar. This approach is an extension of the study of the effect of pressure on the reaction of horseradish peroxidase with hydrogen peroxide which was carried out in water and at room temperature (Ralston et al. 1981, 1982).

2. Materials and methods

2.1. Proteins and reagents

Horseradish peroxidase, type VI, was purchased from Sigma (St. Louis, Mo.) and consists almost exclusively of the C isoenzyme (Marklund et al. 1974). Concentrations were calculated using $\varepsilon_{403} = 102.3$ mM^{-1} (Schonbaum and Lo 1972). Hydrogen peroxide (H_2O_2 "Perhydrol 30%") was purchased from Merck and its concentration determined spectrophotometrically using an absorption coefficient of $39.4\,M^{-1}\,cm^{-1}$ at 240 nm (Nelson and Kiesow 1972). Ethylhydroperoxide (ethyl peroxide) and cytochrome *c* peroxidase were generously provided by Prof. Paul (University of Umeå, Sweden) and Prof. T. Yonetani (University of Pennsylvania), respectively. The ethylhydroperoxide concentration was determined spectrophotometrically using the formation of compound I of the cytochrome *c* peroxidase reactions (Yonetani 1976).

Reagents were obtained from E. Merck (Darmstadt, West Germany). *Tris*-HCl was chosen as buffer since its hydrogen concentration is almost pressure-independent (Neuman et al. 1973). The pH (or paH which takes account of the activity of H^+ in hydro-organic solvents; Douzou 1977) was estimated according to the values published elsewhere (Douzou 1977; Douzou and Balny 1978). For example, a *Tris* buffer of pH 7.5 at 20 °C will have, in the presence of 60% DMSO, paH values of 6.6, 7.3 and 8.4 at 20, 0 and −25 °C, respectively. This paH variation is acceptable since k_+ (see below) is pH independent between pH 5 and 10 (Kato et al. 1984).

2.2. Stopped-flow measurements

Kinetic measurements at atmospheric pressures were made with a stopped-flow apparatus adapted to cryoenzymic conditions built in this laboratory (Markley et al. 1981) and adapted to an Aminco DW 2 spectrophotometer. The dead time of the apparatus is less than 5 ms, the optical pathlength 1 cm and the optical slit 5 nm. Data were recorded by monitoring absorbance changes against time in the dual mode of the Aminco, i.e. the difference of absorbance between 403 and 450 nm where the difference between the absortivities of the enzyme and compound I is at its greatest (Kato et al. 1984).

Care is needed in the interpretation of stopped-flow experiments carried out under cryoenzymic conditions. We have already discussed this problem (Markley et al. 1981) and here we carried out the required test experiments to eliminate artifacts.

2.3. High pressures kinetic measurements

Experiments at high pressures and low temperatures were made using a high pressure device developed in this laboratory (Balny et al. 1984) and the kinetics were recorded in the same way as at atmospheric pressures. Both stopped-flow apparatuses were controlled thermostatically to ±0.1 °C.

2.4. Treatment of data

From the results at low temperatures (see Results), it appears that the binding of peroxide to peroxidase to form compound I is a two steps process:

$$E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^*S\,, \quad \text{(scheme 1)}$$

where E is peroxidase and E*S compound I. K_1 is an association constant which describes a rapid equi-

librium for the formation of the collision complex ES which is then transformed by a slower process to E*S. This process may be the result of a change in state of the active site heme group (e.g. involving the departure of a water molecule; Jones and Dunford 1977) or it may be due to a conformational change of the protein (see Discussion). When $[S] \gg [E]$, the kinetics of formation of E*S is exponential with

$$k_0 = k_2 \cdot K_1 \cdot [S]/(K_1 \cdot [S] + 1) + k_{-2}$$

when $[S] \ll 1/K_1$, $k_0 = k_+ \cdot [S] + k_{-2}$ with $k_+ = k_2 \cdot K_1$.

At very low [S] it appears that k_{-2} is so small that it can be neglected and we can write:

$$k_0 = k_2 \cdot K_1 \cdot [S]/(K_1 \cdot [S] + 1)$$
$$= k_+ \cdot [S] \quad \text{at} \quad [S] \ll 1/K_1 .$$

Thus, by studying k_0 as a function of [S] one can obtain k_2 and K_1, or only k_+ if a saturation plateau in k_0 cannot be attained (rapidity, small K_1).

The thermodynamic parameters pertaining to the equilibrium constant K_1 were calculated from the van't Hoff equation:

$$\Delta G_1 = -RT \cdot \ln K_1$$

under standard conditions (i.e. $[E] = [S] = [ES] = 1\,M$). We obtain ΔH_1 and ΔS_1 by fitting ΔG_1 as a function of T using $\Delta G_1 = \Delta H_1 - T \cdot \Delta S_1$.

The thermodynamic parameters concerning the kinetic constant k_2 were considered within the transition state theory (e.g. Glasstone et al. 1941):

$$ES \underset{}{\overset{k_2}{\rightleftarrows}} ES^{\neq} \overset{k_{-2}}{\rightleftarrows} E^*S$$

with $G_2^{\neq} = -RT \cdot \ln (k_2 h/kT)$ where h is Planck's constant and k the Boltzmann constant. $\Delta G_2^{\neq}$ represents the variation in standard free enthalpy when ES is transformed to $ES^{\neq}$.

We let $\Delta G_+^{\neq}$ represent the thermodynamic parameter associated with k_+; this is equal to $\Delta G_1 + \Delta G_2^{\neq}$ and thus describes the properties of $ES^{\neq}$ with respect to the ground state E + S.

Probably the most rigorous way of presenting the data as a function of the temperature is to use the relationship of Keleti (1983), namely:

$$\Delta G^{\neq} = f(T) = \Delta H^{\neq} - T \cdot \Delta S^{\neq} .$$

Thus, in cases were the thermodynamic parameters $\Delta H^{\neq}$ and $\Delta S^{\neq}$ vary little with the temperature, by plotting $\Delta G^{\neq}$ versus T one obtains a straight line of slope $\Delta S^{\neq}$ and intercept $\Delta H^{\neq}$.

In addition to carrying out temperature dependence studies, we also varied another intensive parameter, the pressure. Here

$$K_1 = A_1 \cdot \exp(-P \cdot \Delta V_1/RT)$$

and

$$k_2 = A_2 \cdot \exp(-P \cdot \Delta V_2^{\neq}/RT) ,$$

where ΔV_1 is the overall variation of the molar volume when E + S forms ES and $\Delta V_2^{\neq}$ when ES forms $ES^{\neq}$.

For k_+, $\Delta V_+^{\neq}$ is the overall variation of volume between E + S and $ES^{\neq}$.

The value for R was taken to be

$$82\ cm^3\ atm \cdot K^{-1} \cdot mol^{-1} \text{ with } 1\ atm = 1.013 \times 10^5\ Pa.$$

All the curves, whether exponential, hyperbolic or linear, were fitted used an Apple IIe computer and the "KINFIT" program of Knack and Röhm (1981). After non-linear regression, this procedure gives the required parameters with standard deviations.

3. Results

3.1. Effect of solvent on the kinetics of formation of compound I above 0 °C

In order to determine the effect of organic solvents on the formation of compound I, comparative experiments were carried out between 20° and 1 °C in the presence or absence of organic solvent, the peroxidase being in the oxidized form. Owing to the rapidity of the binding process at high peroxide concentrations, we mainly used ethylhydroperoxide (ethyl peroxide) as substrate. With this substrate, the kinetics are $\cong 4.5$ fold slower than with hydrogen peroxide (Ohlsson et al. 1984). Experiments were carried out using 5 to 7 μM peroxidase and 8 to 70 μM ethyl peroxide.

In aqueous buffer (0.04 M *Tris*) at 20° or 1 °C, substrate dependence curves of k_0 were linear up to at least 75 s^{-1}: it was difficult to study faster reactions because of the dead time (about 3 ms) of our stopped-flow apparatus. From these substrate dependence curves, the slopes give the second order rate constant k_+ and the values obtained are summarized in Table 1. Also given in Table 1 are estimates for the enthalpy of activation $\Delta H^{\neq}$ pertaining to k_+; these are unusually low for this type of constant. Our values are close to published work (Ohlsson et al. 1984); any differences can be probably explained by medium variations and by the isoenzyme composition of the enzyme used (the predominant isoenzyme in the commercial preparation we used is the C2 type).

We next studied the effect of organic solvents on the substrate binding kinetics of peroxidase. The solvents we chose were DMSO (60%, v/v) and methanol (50%, v/v). We discarded ethylene glycol as this

Table 1. Values and thermodynamic parameters for K_1, k_2 and $k_+ = K_1 \cdot k_2$ under different experimental conditions. The substrate was EtOOH. For other details, see the text

	Temperature [in °C]	$K_1 \times 10^{-3}$ [M^{-1}]	k_2 [s^{-1}]	$k_+ \times 10^{-4}$ [$M^{-1} s^{-1}$]
60% DMSO	19.5			11.8 ± 0.1 [a]
	10			6.3 ± 0.1 [a]
	0	0.073 [b]	330 [b]	2.4 ± 0.1 [a]
	− 10.5	0.33 ± 0.03	35 ± 2	1.15
	− 14	0.39 ± 0.06	17 ± 3	0.65
	− 20	1.9 ± 0.2	1.4 ± 0.1	0.25
	− 25	5 ± 0.3	0.19 ± 0.02	0.095
	− 29.5	6.5 ± 0.6	0.12 ± 0.01	0.080
	ΔH or ΔH^+ in kJ · mol^{-1}	− 95 ± 10	170 ± 18	60 ± 4
	ΔS or ΔS^+ in J · K^{-1} · mol^{-1}	− 310 ± 45	440 ± 70	60 ± 12
50% methanol	19.5			38 ± 2 [a]
	− 17.5	0.11 ± 0.02	660 ± 70	7.25
	− 22.5	0.19 ± 0.05	260 ± 20	4.95
	− 29.5	1.4 ± 0.1	28 ± 2	3.90
	− 37.5	3.4 ± 0.4	3.2 ± 0.3	1.10
	ΔH or ΔH^+ in kJ · mol^{-1}	− 90 ± 13	134 ± 6	30 ± 4
	ΔS or ΔS^+ in J · K^{-1} · mol^{-1}	− 320 ± 50	340 ± 25	− 35 ± 15
Water	19.5			121 ± 2 [a]
	1			108 ± 2 [a]
	ΔH^+ in kJ · mol^{-1}			9 ± 1
	ΔS^+ in J · K^{-1} · mol^{-1}			− 125 ± 15

[a] Values obtained from the slope of k_0 versus EtOOH concentration (e.g. Fig. 1 A)

[b] Extrapolated from the data plots in Fig. 2

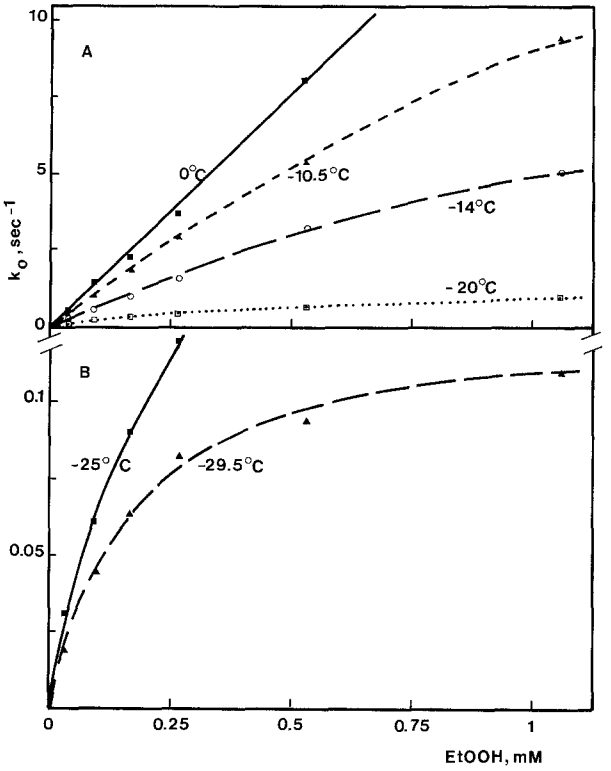

Fig. 1 A and B. Dependence of k_0 on the concentration of EtOOH in 0.04 *M Tris*, paH = 8.7 in 60% (v/v) DMSO at different temperatures. [HRP] = 6.5 μ*M*. **A**: 0 (■), − 10.5 (▲), − 14 (○), − 20 °C (□); **B**: − 25 (■) and − 29.5 °C (▲)

solvent often contains trace amounts of peroxides which would interfere with the formation of compound I.

Both solvents had the effect of greatly reducing k_0 which enabled us to carry out experiments up to 1 m*M* ethyl peroxide. However, the substrate dependence curves remained linear for temperatures above 0 °C (Fig. 1) and from these the k_+ values were obtained (Table 1).

3.2. Effect of temperature on the formation of compound I

The kinetics of the formation of compound I with ethyl peroxide as substrate were studied as a function of temperature down to − 30 °C in 60% DMSO and to − 38 °C in 50% methanol.

With 60% DMSO as cryosolvent, the substrate dependence curves showed an increase in curvature as the temperature was decreased and at − 29.5 °C a saturation plateau was attained (Fig. 1). Thus, at low temperatures we have good evidence that the formation of compound I involves two steps (Scheme 1): a rapid equilibrium (K_1) followed by a slower process (k_2, k_{-2}). Values were obtained for K_1 and k_2 in the temperature range − 10° to − 30 °C (Table 1) but k_{-2} was too small to be determined (e.g. at − 30 °C, see Fig. 1 B). The dependence of ΔG for K_1 and k_2 on the temperature is shown in Fig. 2 (see Treatment of data). The dependence is linear in both cases and the values obtained for ΔS and ΔH are summarized in Table 1.

From the values of K_1 and k_2 obtained at low temperatures one can calculate the composite constant $k_+ = K_1 \cdot k_2$. At temperatures above − 10 °C only k_+ can be obtained: we note that there is a good continuity in the ΔG between the two temperature regions (Fig. 2).

The results obtained in 50% methanol are qualitatively very similar to those obtained in 60% DMSO (Table 1).

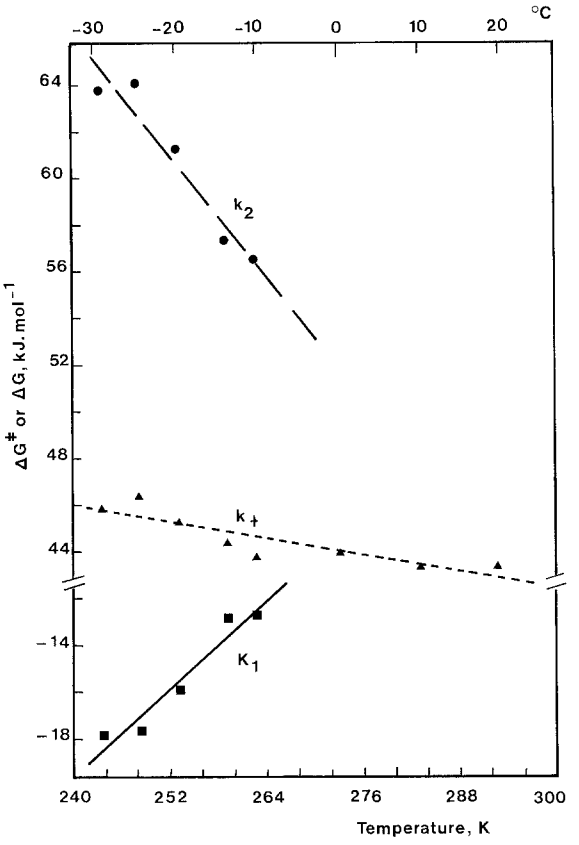

Fig. 2. Plot of $\Delta G^{\neq}$ or ΔG versus T for the three constants K_1 (■), k_2 (●) and k_+ (▲) in 60% DMSO at atmospheric pressure. For experimental conditions, see legend to Fig. 1

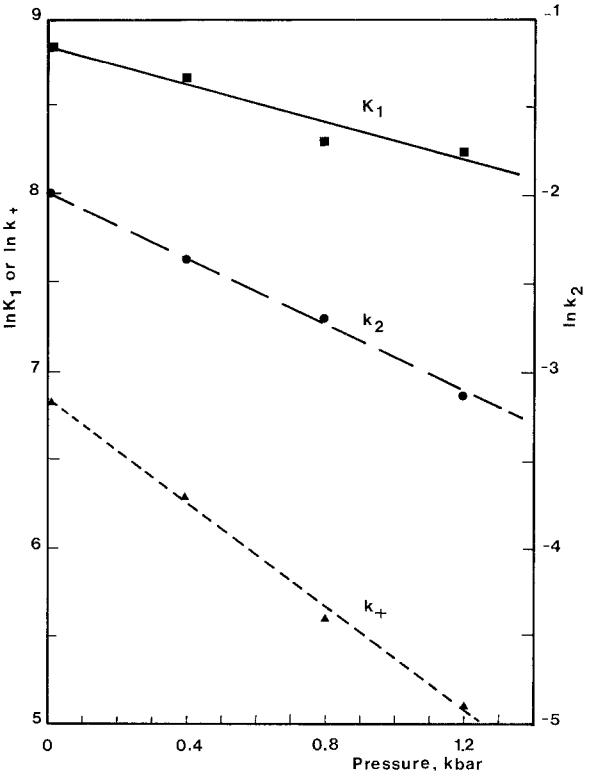

Fig. 3. Dependence of K_1 (■), k_+ (▲), left hand scale and k_2 (●), right hand scale, on pressure in 60% DMSO at −23 °C. The lines were computer fitted. For full details, see text

3.3. Formation of compound I with H_2O_2 as the substrate

At low temperatures, with 60% DMSO or 50% methanol as the cryosolvent, we obtained saturation kinetics with H_2O_2 as the substrate. From the k_0 versus H_2O_2 concentration curves, K_1 and k_2 were calculated; the values obtained in the two solvents are summarized in Table 2 together with the data with ethyl peroxide as substrate.

Table 2. Comparative values at −29.5 °C of K_1, k_2 and k_+ in two solvents systems for two substrates. For other experimental conditions, see text

Solvent	60% DMSO		50% MeOH	
Substrate	EtOOH	H_2O_2	EtOOH	H_2O_2
$K_1 \times 10^{-3}$ (M^{-1})	6.5 ± 0.6	3.3 ± 0.3	1.4 ± 0.1	0.9 ± 0.1
k_2 (s^{-1})	0.12 ± 0.01	0.56 ± 0.02	28 ± 2	330 ± 30
$k_+ \times 10^{-4}$ [a] ($M^{-1}\,s^{-1}$)	0.080	0.18	3.90	30

[a] Calculated, $k_+ = K_1 \cdot k_2$

3.4. The effect of pressure on the kinetics of formation of compound I

k_0 was obtained as a function of the pressure (up to 1.2 kbar) under various conditions. In all cases k_0 decreased as the pressure was increased.

These experiments were carried out in the high-pressure stopped-flow apparatus; with this apparatus at atmospheric pressures the value for k_0 agreed well with that obtained using our conventional stopped-flow apparatus under identical conditions.

To determine the effect of pressure on K_1 and k_2, one must determine the dependence of k_0 on the substrate concentration (here ethyl peroxide) at different pressures. We were able to do this only at −23 °C in 60% DMSO (Fig. 3): above −23 °C saturation kinetics were difficult to attain. At lower temperatures, the solvent froze above 1.2 kbar so it was difficult to obtain an accurate value for $\Delta V^{\neq}$. Instead, we explored the $\Delta V^{\neq}$ of k_+ as a function of the temperature in water and 60% DMSO (Fig. 4) and our results are summarized in Table 3.

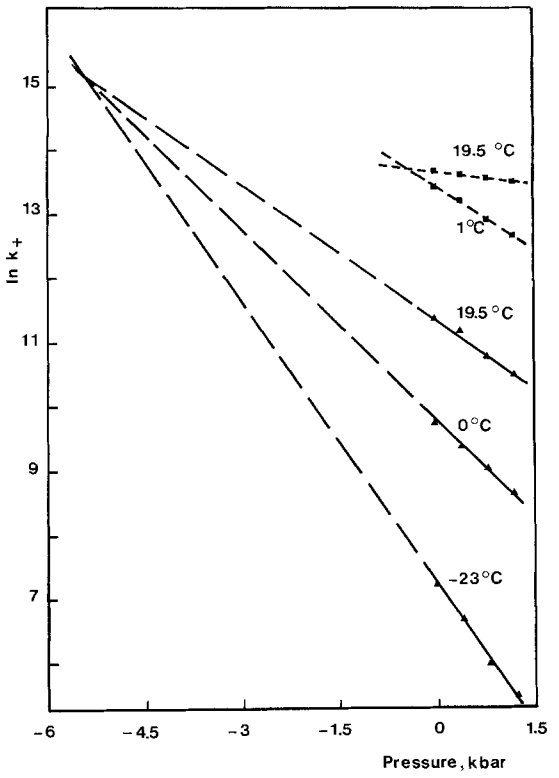

Fig. 4. Pressure dependence of k_+ in water (■) and in 60% DMSO (▲), at different temperatures. The lines were computer fitted. For experimental conditions, see text

Table 3. Activation volumes (in ml · mol^{-1}) for K_1, k_2 and k_+ under various experimental conditions. Substrate: EtOOH

Experimental conditions [a]		Nature of kinetic constants [b]		
Solvent	Temperature [°C]	K_1 [ΔV]	k_2 [$\Delta V^{\neq}$]	k_+ [$\Delta V^{\neq}$]
60% DMSO	+ 19.5			18 ± 1.5
	0			20.5 ± 1
	− 23	10.8 ± 1.5	19.2 ± 0.8	30 ± 1.5
H_2O	+ 19.5			3 ± 0.5
	1			14.6 ± 1.5

[a] For full details, see text

[b] Values calculated as described in Materials and Methods. Means ± SD are obtained by computer fitting of ln (kinetic constants) as a function of pressure

4. Discussion

4.1. Evidence that horseradish peroxidase binds peroxides in a two-step process at neutral pH

It was by carrying out peroxide concentration dependence experiments under cryoenzymic conditions that we were able to show that the binding kinetics reach a plateau at neutral pH (Fig. 1). The relationship was hyperbolic which strongly suggests that the binding of peroxide to horseradish peroxidase is a two step process (Scheme 1). Job and Dunford (1978) came to the same conclusion from their experiments carried out with the high pH form of the enzyme. A two step binding process for peroxide has been found with the closely related catalase (Jones and Suggett 1968).

4.2. Step 1: formation of the collision complex

The first step on the reaction pathway of an enzyme is the formation of the collision complex ES (Scheme 1). This is generally considered to be a rapid equilibrium (i.e. $k_2 \ll k_1$, k_{-1}; Gutfreund 1972). It is noteworthy that in none of our kinetic curves for the formation of compound I could we detect a transient lag phase (a similar situation obtains in the binding kinetics of ATP to myosin ATPase, Barman et al. 1983) and this confirms a rapid equilibrium situation with the peroxidase under the various experimental conditions used.

From Table 2, one can see that at a given temperature, the effect of changing the solvent from 60% DMSO to 50% methanol is to change K_1 by a factor of 4.5. This relatively small effect is of interest as these solvents are very different. Thus, whereas methanol has little effect on the structure of water, DMSO is a potent "structure breaker" and much heat is evolved when it is mixed with water. The relatively small effect of the solvent on K_1 is illustrated further by comparing ΔH and ΔS in the two solvents (Table 1).

This is in striking contrast to the association constant describing the collision complex of myosin ATPase (Barman et al. 1983), and may be due to the fact that unlike ATP, peroxide binding does not involve electrostatic interactions and is therefore insensitive to the dielectric constant of the solvent environment.

We further note that there is little difference in K_1 for H_2O_2 and ethyl peroxide (Table 2).

4.3. Step 2: formation of compound I

There is considerable evidence that a protein isomerization follows the initial binding of a substrate to an enzyme. Examples of this induced fit process (Koshland 1958) are hexokinase (Anderson and Steitz 1975) and myosin ATPase (Barman et al. 1983). An induced fit implies a substrate induced conformational change of the enzyme *protein* and

there is at present no evidence for this with horseradish peroxidase.

The spectral changes observed on the binding of peroxides presumably involves some movements within the peroxidase molecule but this may involve only the heme moiety. Thus it is known that peroxides bind to certain hemes in two steps (e.g. deuterohemin, Portsmouth and Beal 1971; deuteroferriheme, Jones et al. 1974) but these processes are considerably slower than with peroxidase. In common with peroxidase, the binding of peroxide leads to a modification of the spectral band of the heme and this appears to be the first directly observable event on the reaction pathway of these systems.

From this, a general mechanism for the reaction of peroxides with peroxidases to form compound I have been described; this involves histidine, arginine and tryptophan residues of the enzyme (Poulos and Kraut 1980, 1982). An aspartic acid residue may be implicated (Dunford and Araiso 1979). However, it is not known if the peroxide substrate causes any of these residues to move with respect to the rest of the protein.

Whereas there is little information on the dynamics of the protein portion of horseradish peroxidase on compound I formation, the dispositions of the heme and iron have been discussed (recent papers are Poulos and Kraut 1980; Fujita et al. 1983; Ohlsson et al. 1984). It has been suggested that when compound I is formed, water is excluded from the active site (Jones and Dunford 1977). These phenomena, however, cannot be interpreted within the induced fit theory.

Finally we note that when in the crystalline state, the closely related cytochrome *c* peroxidase can be converted to its compound I with retention of crystallinity (Poulos and Kraut 1982).

From the above we assume here that the transformation ES $\rightleftarrows$ E*S (Scheme 1) is an isomerization without specifying which portion of the peroxidase molecule is involved.

It appears that a key property of step 2 is its irreversibility, a phenomenon observed with both H_2O_2 and ethyl peroxide. Thus, the extrapolated value for k_+ at [substrate] = 0, is too low to be measured (k_{-2}; see Fig. 1). ΔG_2 for step 2 is, therefore, negative and large and the overall binding process is strongly directed towards compound I. The irreversibility of the overall reaction is, therefore, the reflection of a low k_{-2}.

Whereas K_1 differs little with H_2O_2 and ethyl peroxide, k_2 varies 5–12 fold, depending on the experimental conditions (Table 2). The specificity of this enzyme is, therefore, largely a manifestation of k_2 rather than K_1.

k_2 is remarkably sensitive to the solvent. For instance, at −30 °C there is a 160 fold difference between 60% DMSO and 50% methanol as the solvent. It appears, therefore, that the type of solvent used has a profound effect on the process described by k_2. That the amplitude of this process (which, of course, manifests itself by an observable spectral change) is large is illustrated further by the values of the thermodynamic parameters pertaining to k_2. Thus, in 60% DMSO, at −30 °C, $\Delta H_2^{\neq} = 170\ \mathrm{kJ \cdot mol^{-1}}$, $\Delta S_2^{\neq} = 440\ \mathrm{J \cdot mol^{-1} \cdot K^{-1}}$ and at −23 °C, $\Delta V_2^{\neq} = 19\ \mathrm{ml \cdot mol^{-1}}$. Such large values suggest large structural rearrangements which need not only be limited to the neighbourhood of the heme but may also involve the hydration shell at the protein-solvent interface. Similar results were obtained for the reaction of cytochrome *c* peroxidase with hydroperoxide and the data were interpreted in terms of protein structural modifications (Balny et al. 1984).

In conclusion, it is clear that the transition ES → compound I described by k_2 is very sensitive to the medium conditions and the nature of the substrate whereas K_1, which describes the formation of the collision complex ES, is very much less sensitive. Therefore, it is clear that the variations with experimental conditions of the overall constant $k_+ (= K_1 \cdot k_2)$ is primarily due to the sensitivity of k_2.

Finally, it is of interest to consider possible values for K_1 and k_2 in water at 20 °C. From the temperature dependence of K_1 in 60% DMSO and assuming that there is no curvature or break, one can estimate that at 20 °C $K_1 = 5\ M^{-1}$. Since it appears that the solvent does not have a large effect on K_1, we can say that under "normal" aqueous conditions $K_1 < 5\ M^{-1}$. This corresponds to a dissociation constant of $> 200\ \mathrm{m}M$ – a high value which would explain the great difficulty in obtaining saturation in k_0 in water at neutral pH. Under the same conditions $k_+ = 1.2 \times 10^6\ \mathrm{M^{-1} s^{-1}}$ which leads to $2.5 \times 10^5\ \mathrm{s^{-1}}$ for k_2 – a value well beyond estimation by classical kinetic methods.

4.4. Thermodynamic considerations

Since we could only carry out temperature dependence studies on K_1 and k_2 over a narrow temperature range, we could not obtain accurate estimates for the thermodynamic parameters pertaining to these constants. However, we were able to study k_+ over a wide temperature range and under different medium conditions – in particular in water and at 19.5 °C. As we have already explained (Treatment of data, above), the thermodynamic properties of k_+ pertain to the activation state $ES^{\neq}$ preceding compound I with respect to the ground state E + S. These

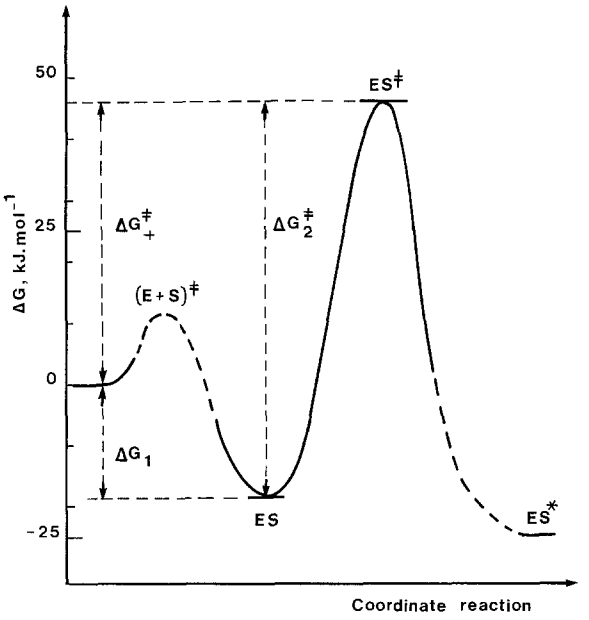

Fig. 5. Energy diagram of the reaction scheme: $E+S \rightarrow ES^{\ddagger} \rightarrow ES^{*}$ for horseradish peroxidase. The values are those in 60% DMSO at −29.5 °C. Dashed curves represent the unknown values. k_1, k_{-1} and k_{-2} are not determined

properties are independent of the energy level of ES (Fig. 5). The discussion which follows is concerned with k_+.

We obtained values for $\Delta H_+^{\ddagger}$ and $\Delta S_+^{\ddagger}$ in three solvents: water, 50% methanol and 60% DMSO (Table 1). In the three solvents, $\Delta H^{\ddagger}$ was 9, 30 and 60 kJ · mol^{-1} and $\Delta S^{\ddagger} = -125$, −34 and + 60 J · K^{-1} · mol^{-1}, respectively. Thus, the peroxidase system is an example of a classical thermodynamic effect, i.e. the compensation effect between the enthalpy and entropy ($-T \cdot \Delta S$) terms. The interpretation of this effect is not obvious; it is probably primarily a reflection of the role of the solvent gain in the macroscopic manifestation of the thermodynamic properties (Lumry and Rajender 1970). It seems unlikely that on passing from water to 60% DMSO the large variation in $\Delta S_+^{\ddagger}$ (from −125 to + 30 J · K^{-1} · mol^{-1}) corresponds to a radical structural change of the enzyme as in both solvents the same reaction mechanism and spectra are observed.

When the pressure dependence of $\Delta G_+^{\ddagger}$ was studied at different temperatures, in water or 60% DMSO, a series of converging lines were obtained (Fig. 4). Obviously, the extrapolations to negative pressures have no physical meaning: they are purely analytical. This behaviour is predicted from the general equation $\Delta G = f(T,P)$ but the physical significance of the point of convergence remains obscure and depends on the reference state for ΔG (Morild 1981). Nevertheless, there is an important practical conclusion of these experiments: $\Delta V_+^{\ddagger}$ is highly temperature sensitive. Thus, at 20 °C in water $\Delta V_+^{\ddagger}$ is practically zero whichever substrate is used (Ralston et al. 1981) whereas at −23 °C in 60% DMSO it is + 30 ml · mol^{-1} (present work). Therefore, as with the changes observed with the other thermodynamic parameters (see above), it seems unlikely that the large change in $\Delta V_+^{\ddagger}$ is due to a change of the actual volume of the protein. The question then, is − which physico-chemical event leading to compound I formation affects the $\Delta V_+^{\ddagger}$ of k_+?

Dunford and Araiso (1979) suggest that during the formation of compound I, an aspartic acid residue is protonated. There is evidence that the addition of a proton to a carboxyl group is associated with a volume change of 10 − 12 ml · mol^{-1}, a process which is rather insensitive to temperature (Azano and Le Noble 1978). Therefore, because of its high temperature sensitivity, it is unlikely that $\Delta V_+^{\ddagger}$ is solely the manifestation of a protonation process.

At present, therefore, it is very difficult to give a precise physical meaning to $\Delta V^{\ddagger}$. Rather, $\Delta V^{\ddagger}$ should be considered as a thermodynamic parameter associated with the reacting species and the solvent environment. Low and Somero (1975) studied the effect of pressure on the kcat of certain enzyme reactions and they suggest that the principal factors in determining $\Delta V^{\ddagger}$ are the movements involved in the masking/unmasking of certain groups at the protein surface.

Such changes in position of chemical groups at many locations on the protein molecule are presumably accompanied by changes in the interaction energies of those groups, or between them and the surrounding water and ionic solutes at the protein-solvent interface. The free energy of activation ($\Delta G_+^{\ddagger}$) would depend on the sum of all these changes involving groups throughout the entire enzyme molecule, and it is obvious from our results in mixed solvents that, by modifying the degree to which the solvent organizes itself around transferred groups, these perturbants may raise or lower $\Delta G_+^{\ddagger}$ and then decrease or increase the rate constant (k) of the step. Such processes would be expected to modify the hydration density of the enzyme and $\Delta V_+^{\ddagger}$ values should reflect such modifications.

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