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ACTIVATION VOLUMES FOR HORSERADISH PEROXIDASE COMPOUND II REACTIONS

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The activation volumes for the reactions of horseradish peroxidase compound II with L-tyrosine, 3-iodo-L-tyrosine, *p*-aminobenzoic acid and ferrocyanide were determined by using a high-pressure stopped-flow technique at 25°C and pH 7. For the tyrosines, the solvent electrostriction accompanying substrate ionization and H^+ transfer from the substituted phenol to a basic group of the enzyme can account for the observed negative activation volumes. For *p*-aminobenzoic acid a simple electron transfer without H^+ transfer appears to occur. The positive activation volume for ferrocyanide may be explained in terms of electron transfer associated with a large change in electrostriction of the inorganic redox couple.

1. Introduction

The peroxidases are enzymes which can utilize hydroperoxides of the type ROOH to oxidize a wide variety of substrates. Most peroxidases are monomeric proteins which contain one molecule of ferriprotoporphyrin IX as a prosthetic group [1]. Two oxidized intermediate species, compound I and compound II, named in their order of appearance in the obligatory steps of the enzymatic cycle, contain two and one oxidizing equivalents, respectively, obtained from the hydroperoxide. The enzymatic cycle of horseradish peroxidase (HRP) may be represented as follows [1]:

$$\underset{[Fe(III)]}{HRP} + H_2O_2 \rightarrow \underset{[Fe(IV)+R^{\cdot}]}{\text{compound I}} + H_2O$$

$$\underset{[Fe(IV)+R^{\cdot}]}{\text{Compound I}} + \text{ferrocyanide} \rightarrow \underset{[Fe(IV)]}{\text{compound II}} + \text{ferricyanide}$$

$$\underset{[Fe(IV)]}{\text{Compound II}} + \text{ferrocyanide} \rightarrow \underset{[Fe(III)]}{HRP} + \text{ferricyanide}$$

where $R^{\cdot}$ represents a free radical species. Thus, compound II is an Fe(IV) species. The reactions of horseradish peroxidase compound II with a variety of substrates have been intensively studied under atmospheric pressure [2–6]. The purpose of the present study is to investigate the effect of high pressure on some of these reactions in the hope of shedding further light upon their mechanisms.

Activation volumes have been shown to be useful as a mechanistic criterion in both organic and inorganic chemistry [7]. They have been used less in biological chemistry. This is due to the fact that, until recently, no fast reaction techniques such as stopped-flow were available for high-pressure studies. The study of the effects of pressure on redox reactions of heme proteins is an almost unexplored field [8]. Studies of hyperbaric oxygenation and dehydrogenase reactions have been reviewed [9], as has the theory of pressure effects on inorganic redox reactions [10].

In the present report, the use of high-pressure stopped-flow to yield volumes of activation for the reactions of compound II with ferrocyanide, *p*-aminobenzoic acid, L-tyrosine and 3-iodo-L-tyrosine is described. An interpretation of the results in terms of proposed reaction mechanisms is offered.

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2. Experimental

2.1. Materials

The horseradish peroxidase (EC 1.11.1.7, donor:hydrogen-peroxide oxidoreductase) was purchased from Boehringer Mannheim Corp. (lot 1399140) as an ammonium sulphate suspension. Dialysis against quintuply distilled water [11] yielded a solution of purity number 3.22 (ratio of absorbances at 403 and 280 nm) [1]. The enzyme concentration was determined at 403 nm using a molar absorption coefficient of $1.02 \times 10^5\ M^{-1}\ cm^{-1}$ [12].

The chemicals were purchased from the following companies: potassium ferrocyanide from Merck-Darmstadt; *p*-aminobenzoic acid from Fisher; L-tyrosine from Eastman Kodak; 3-iodo-L-tyrosine from Calbiochem and H_2O_2 from Fluka. The chemicals were used without further purification. The concentrations of all solutions were determined by weight with the exception of H_2O_2 for which the same standardization procedure was performed as has been previously described for an alkyl hydroperoxide [3].

2.2. Apparatus and methods

All high-pressure kinetic experiments were performed at 25.0 ± 0.2°C on a stopped-flow apparatus specifically designed for this purpose. The entire unit including drive and stop syringes, mixing and observation chambers was inserted into a thermostatically controlled, oil-containing vessel in which pressure (up to a maximum of 1500 atm) was applied. A more detailed description is available [13]. Absorbance measurements were performed on a Cary 118 spectrophotometer and the pH was measured with a Radiometer model 26 pH meter in conjunction with a Radiometer/Copenhagen electrode. All solutions were 0.10 ionic strength in potassium nitrate and 0.01 ionic strength in buffer. Tris buffer was used for all experiments, except in the case of *p*-aminobenzoic acid where phosphate buffer of pH 7.0 was used. Changes in pH in the latter buffer with pressure may be ignored in the case of *p*-aminobenzoic acid, since the rate constant is pH independent in this region. For the other substrates, the rate constant is very pH dependent near neutrality and the use of Tris buffer was necessary to ensure no change in pH with pressure [14]. Nevertheless, the buffering capacity of Tris buffer is limited near pH 7, resulting in a slight variation in pH for each of the remaining substrates.

Compound II was prepared by the addition of a 1.1 M equivalent of ferrocyanide to the enzyme followed by the subsequent addition of a 0.9 M equivalent of H_2O_2 [1].

The observation chamber enzyme concentrations were 2.5 μM. The excess of substrate, always at least 10-fold, ensured pseudo-first-order conditions. The decomposition of compound II was followed at 425 nm. Average first-order rate constants were calculated from the results of three or four traces recorded for each reaction under a specific set of conditions.

3. Results and discussion

The dependence on pressure of the rate constants for (A) ferrocyanide, (B) 3-iodo-L-tyrosine, (C) *p*-aminobenzoic acid and (D) L-tyrosine is shown in fig. 1. The data are summarized in table 1. The values for the second-order rate constants at 1 atm correspond well with reported values [2–6]. No literature value for the rate constant of the reaction of compound II with monoiodotyrosine is available. The activation volumes are calculated from the slopes of $\ln k$ versus p by means of the equation,

$$d \ln k/dp = -\Delta V^{\ddagger}/RT.$$

Available evidence in the literature indicates that ferrocyanide and *p*-aminobenzoic acid react by similar mechanisms in which the enzyme acts as an acid catalyst [2–5]. At neutral pH and in alkaline solution, the influence of a distal group on the enzyme with a pK_a value of 8.6 is dominant. There is no ionization of the substrates in this pH region.

The phenols, on the other hand, ionize in the alkaline region and their anionic form appears to be unreactive [6,15]. Therefore, the phenolic H^+ appears to be essential for reaction. A simple

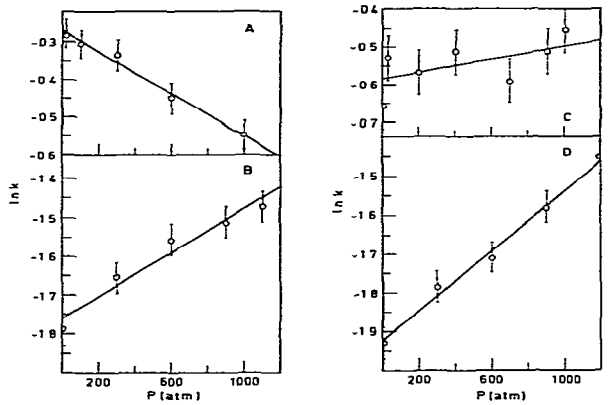

Fig. 1. Dependence of $\ln k$ (k expressed as pseudo-first-order constant, units s^{-1}) on pressure for the following reactions with horseradish peroxidase compound II: (A) ferrocyanide, (B) 3-iodo-L-tyrosine, (C) *p*-aminobenzoic acid, (D) L-tyrosine. A particular first-order constant may be converted to a second-order constant by dividing k by the following concentrations: ferrocyanide, 5.9×10^{-5} M; 3-iodo-L-tyrosine, 1.24×10^{-4} M; *p*-aminobenzoic acid, 1.46×10^{-3} M; L-tyrosine, 1.43×10^{-4} M.

calculation showed that no correction was necessary for the influence of substrate ionization [16] on the observed kinetics as a function of pressure. The pK values of iodotyrosine and tyrosine lie outside the pH range of our experiments and are therefore not affected by pressure.

The reaction of compound II with ferrocyanide, in contrast to the other substrates, has a positive activation volume (+6.7 ml). The reaction can be written as

$$\underset{[Fe(IV)]+[Fe(II)]}{\text{Compound II + ferrocyanide}} \rightarrow \underset{[Fe(III)]+[Fe(III)]}{\text{HRP + ferricyanide}}$$

The volume change of this reaction is expected to be large and positive, mostly due to the change in partial molar volumes of the iron cyanides [17]. Electrostriction is reduced as compound II gains an electron and the formal oxidation state changes from IV to III, simultaneously with the conversion of Fe(II) to Fe(III) in the substrate. A positive volume of activation is therefore expected, as observed. A theoretical calculation of the activation volume is not possible without the knowledge of the total reaction volume [18].

p-Aminobenzoic acid yields a small negative activation volume which is in accord with an electron transfer without large changes in overall electrostriction [18].

The tyrosines have larger negative activation volumes, −7.0 ml/mol for monoiodotyrosine and −9.4 ml/mol for tyrosine. Ionization of a phenol results in a volume decrease of −19 ml/mol [16]. For the substituted phenols the volume changes due to electron transfer are assumed to be small as for *p*-aminobenzoic acid, compared to the volume contributions from H^+ transfer. Two different possibilities then may be envisaged to account for the observed activation volumes: (1) fractional ionization of the phenol in the transition state with partial H^+ transfer to a neutral basic group on the enzyme, since the contribution of the latter process is small [16]; (2) total ionization of the phenol and total H^+ transfer to a negative group on the enzyme. The latter process leads to a volume change of approx. +10 ml/mol [16]. Therefore, the combined volume changes for phenol ionization and H^+ transfer would account for the observed results. It is not possible at present to distinguish between these two possibilities. We note, however, that the role of a distal carboxyl group in compound I formation appears to be established [19]. In either case, the picture that emerges is of an enzyme activated by H^+ transfer from the tyrosine substrates.

Thus, the measured activation volumes for compound II reaction of horseradish peroxidase appear to be in accord with and lend support to the published mechanisms.

Table 1

Rate constants at 1 atm and activation volumes for reactions of horseradish peroxidase compound II at 25°C, 0.11 ionic strength and the indicated pH values

Substrate	$k(M^{-1}s^{-1})$	$\Delta V^{\ddagger}$ (ml/mol)	pH
Ferrocyanide *	1.3×10^4	+6.7	6.94
3-Iodo-L-tyrosine	1.4×10^3	−7.0	7.33
p-Aminobenzoic acid	3.5×10^2	−2.5	7.00
L-Tyrosine	1.0×10^3	−9.4	7.40

* Extrapolated value from fig. 1.

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