Formation and properties of dimeric recombinant horseradish peroxidase in a system of reversed micelles

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Wild-type recombinant horseradish peroxidase purified and refolded from *Escherichia coli* inclusion bodies has been studied in the system of bis(2-ethylhexyl)sulphosuccinate sodium salt (Aerosol OT)-reversed micelles in octane. In contrast with native horseradish peroxidase the wild-type recombinant enzyme forms dimeric structures as judged by sedimentation analysis. Peroxidase substrates affect the equilibrium between monomeric and dimeric enzyme forms. The dependence of the catalytic activity of recombinant peroxidase on the degree of hydration of the surfactant exhibits two maxima with pyrogallol, *o*-phenylenediamine, guaiacol and *o*-dianisidine, with different ratios of activities for the first and second maxima. The differences in activities of monomeric and dimeric forms of the recombinant horseradish peroxidase provide evidence for active-site screening in dimeric forms. This has been used to model a dimeric structure of recombinant horseradish peroxidase with the screened entrance to the active site. In the model structure obtained, three of eight glycosylation sites were screened. This might explain the absence of dimeric structures in native enzyme peroxidase. The system of reversed micelles provides, for the first time, evidence for the formation of dimeric structures by recombinant plant peroxidase with an altered substrate specificity compared with the native enzyme. Thus one can assume that haem-containing peroxidases in general are able to form dimeric structures.

INTRODUCTION

Horseradish peroxidase (HRP) belongs to the superfamily of plant peroxidases (donor:H_2O_2 oxidoreductase, EC 1.11.1.*X*, where *X* depends on the biological reducer). The superfamily includes enzymes with various functions, and attempts to classify them continue. In accordance with the widespread classification [1], the superfamily is subdivided into three classes based on sequence alignments and biological origin. The first prokaryotic class represents microbial enzymes, i.e. bacterial peroxidase–catalases (EC 1.11.1.6) and yeast cytochrome *c* peroxidase (EC 1.11.1.5), as well as plant ascorbate peroxidases (EC 1.11.1.11). The distinguishing feature of these non-glycosylated peroxidases is their ability to form dimeric and tetrameric structures.

The second and third classes (EC 1.11.1.7) include fungal and plant peroxidases respectively. These enzymes are monomeric glycoproteins with four conserved disulphide bridges and two conserved calcium sites. The whole set of crystallographic data on the tertiary structures of cytochrome *c* peroxidase [2], dimeric pea ascorbate peroxidase [3], lignin and manganese-dependent peroxidase from *Phanerochaete chrysosporium* [4,5] and peanut peroxidase [6] and preliminary results reported on the recombinant HRP [7] make evident a common motif in folding of all haem-containing peroxidases: ten basic α-helices forming two domains with the non-covalently but firmly entrapped haem.

Thus among the plant peroxidase superfamily only prokaryotic non-glycosylated peroxidases were shown to form dimeric structures *in vivo*, which is in contrast with glycosylated plant and fungal peroxidases [3]. However, this particular feature is very common for the superfamily of mammalian peroxidases, where myeloperoxidase, thyroid peroxidase and prostaglandin H synthase [8–10] function as dimeric structures. The physiological significance of this phenomenon is still unclear.

Progress in HRP gene cloning and expression in *Escherichia coli* [11,12] allows us to produce a non-glycosylated and catalytically active protein. This work describes new features of the recombinant HRP revealed in the system of bis-(2-ethylhexyl)-sulphosuccinate sodium salt (Aerosol OT)-reversed micelles in octane. Micellar enzymology is a novel approach to the study of the properties of biomolecules, and enzymes in particular. Its application reveals the enzyme's tendencies to form superactive conformations and supramolecular structures (reviewed in [13–17]). The results presented in this paper provide a new look into structure–function relationships in peroxidase catalysis.

EXPERIMENTAL

Materials

Native HRP RZ 3.0 was purchased from Yarinvest Medical International (Russia). The enzyme concentration was determined spectrophotometrically, using a molar absorption coefficient of 102000 $M^{-1} \cdot cm^{-1}$ at 403 nm [18].

Recombinant HRP was produced in *E. coli* inclusion bodies and refolded in accordance with the protocol developed earlier [12]. The preparation was homogeneous as judged by SDS/PAGE and HPLC (molecular mass 34 kDa). The protein concentration was determined spectrophotometrically as described in [19].

Pyrogallol (Serva) was additionally purified by vacuum sublimation. Guaiacol, *o*-phenylenediamine, *o*-dianisidine, ammonium 2,2′-azino-bis-(3-ethyl-benzothiasoline-6-sulphonate and Aerosol OT were purchased from Sigma (St. Louis, MO, U.S.A.) and used without additional purification. Because Aerosol OT preparation contained 0.4 mol of water per mol of surfactant, according to infrared spectroscopy data, this value was taken into account when the total amount of water in the micellar system was calculated. H_2O_2 and n-octane were purchased from Reakhim (Moscow, Russia). The latter was purified as described in [20]. H_2O_2 concentration was determined spectrophotometri-

Abbreviations used: Aerosol OT, bis-(2-ethylhexyl)sulphosuccinate sodium salt; HRP, horseradish peroxidase.

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Table 1 HRP enzymic assay conditions in water and micellar solutions

Donor substrate	Wavelength (nm)		Molar absorption coefficient ($M^{-1} \cdot cm^{-1}$)		Buffer	pH	Donor substrate concentration range (mM)
	Water	Octane	Water	Octane			
Pyrogallol	420	420	4500 [22]	4400 [26]	20 mM Tris/acetate	6.0	0.40–4.00
Guaiacol	436	470	3000 [23]	2000	30 mM citrate/0.05% $CaCl_2$	5.0	4.50–18.00
o-Dianisidine	460	385	31000 [24]	24500	20 mM citrate	6.0	0.08–0.25
o-Phenylenediamine	420	450	31250 [25]	66700	20 mM phosphate/citrate	6.0	0.01–0.12

cally, using a molar absorption coefficient of $72.7\,M^{-1} \cdot cm^{-1}$ at 230 nm [21].

Methods

All kinetic experiments were performed on a Philips PU-8630 spectrophotometer with a thermostatically controlled cell holder at 25 °C. The concentrations of native and recombinant HRP were 0.06 and 0.4–4 nM respectively.

In a typical experiment, 30–90 μl of a buffer solution and aliquots (2–5 μl) of the substrate and enzyme solutions were solubilized in 2 ml of 0.1 M Aerosol OT in octane. The mixture was shaken vigorously after each reagent addition to get an optically transparent solution. Reactions were initiated by the addition of H_2O_2. The following stock solutions of the donor substrates were used: 4 M pyrogallol and 40 mM guaiacol in acetone, 0.13 M *o*-dianisidine in 60% (v/v) ethanol, and 0.24 M *o*-phenylenediamine in acetonitrile. The assay protocols for pyrogallol [22], guaiacol [23], *o*-dianisidine [24] and *o*-phenylenediamine [25] were modified to use similar buffer solutions and pH values.

To monitor product accumulation, the optimal wavelength and the corresponding molar absorption coefficients were initially determined for each substrate in the the system of Aerosol OT-reversed micelles in octane at different degrees of hydration of the surfactant, w_0 = [water]/[Aerosol OT]. The assay conditions (buffer solutions, wavelengths, molar absorption coefficients) for a number of electron donors used in this work are summarized in Table 1.

The maximal reaction rates were calculated as described elsewhere [26] by varying the concentration of donor substrates at the fixed saturating concentration of H_2O_2.

Sedimentation analysis was performed on a Spinco model E analytical ultracentrifuge (Beckman, Irvine, CA, U.S.A.) equipped with a photoelectric scanning device, a monochromator and a multiplexor, with 12 mm bisection cells and an An-G-Ti rotor at 100000 ***g***. Scanning was performed at 403 nm. Sedimentation coefficients were calculated from the experimental data as described earlier [27].

Dimer modelling and structure optimization were performed with the Insight II (version 95.0) program (Biosym/MSI, San Diego, CA, U.S.A.). The unavailability of the crystal structure coordinates of recombinant HRP led us to use the model structure previously created by means of homology modelling on the basis of the crystal structure of peanut peroxidase [28]. Models of dimers were created by means of various orientations of two HRP molecules followed by energy relaxation of the system (2000-step energy minimization, then molecular dynamics modelling for 50 ps and again 2000-step energy minimization). Relaxation calculations were performed with the AMBER force-field [29].

RESULTS AND DISCUSSION

Most of the enzymes studied in the systems of reversed micelles exhibit a characteristic bell-shaped dependence of their catalytic activity on the degree of surfactant hydration [13–17]. Variations in this parameter are accompanied by changes in the size of the

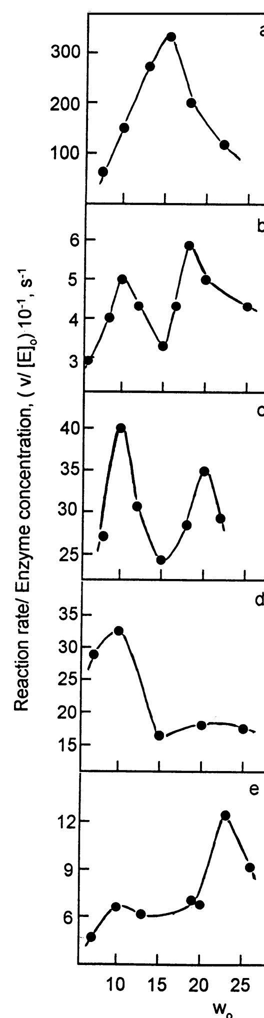

Figure 1 Dependence of catalytic activity, $V/[E]_0$ (reaction velocity/total enzyme concentration) of native (a) and recombinant (b–e) HRP, in the oxidation of pyrogallol (a,b), guaiacol (c), *o*-dianisidine (d) or *o*-phenylenediamine (e) by H_2O_2 in Aerosol OT-reversed micelles in octane, on the degree of hydration, w_0, of the surfactant

Table 2 Sedimentation coefficients of the reversed micelles of 0.1 M Aerosol OT in octane containing 1 μM recombinant HRP in the presence and the absence of peroxidase substrates

Theoretical values were calculated by the method described in [24]. Sedimentation coefficients for unfilled micelles are higher for higher w_0 values.

		$10^{13} \times$ Sedimentation coefficient			
			$w_0 = 20$		
Donor substrate	Concentration (nM)	$w_0 = 10$ Monomer	Monomer	Dimer	Active centres in dimer form (%)
Theoretical values					
For 34 kDa form	–	22	25	–	–
For 68 kDa form	–	–	–	31	–
Experimental values					
Without substrate	–	22	24	29	50
Pyrogallol	10	22	23	29	38
Guaiacol	100	22	25	33	53
o-Dianisidine	100	22	24	29	28
o-Phenylenediamine	50	23	24	32	62

inner water cavity of reversed micelles [30,31]. Maximal enzyme catalytic activity in reversed micelles is observed at a particular degree of surfactant hydration, namely when the size of the inner cavity corresponds to that of the entrapped protein (general regularities of the phenomenon are discussed in [17]). This was previously observed for native HRP assayed with aromatic alcohols and amines [26,32,33]. The micellar matrix also enhances the catalytic activity of native HRP compared with the enzyme in aqueous solution owing to its interaction with HRP oligosaccharide chains [26]. Figure 1(a) illustrates a typical dependence of catalytic activity of native HRP (expressed as a ratio of the maximum reaction rate to the enzyme concentration) on degree of hydration for pyrogallol oxidation by H_2O_2 in the system of Aerosol OT-reversed micelles. The dependence has one maximum with the optimum of HRP catalytic activity at $w_o = 15$ when the protein size and the micelle inner cavity fit each other closely. The micellar matrix in the case of native glycosylated HRP results in the stabilization of a superactive conformation, probably owing to the interaction between hydrophilic HRP oligosaccharides and the hydrophilic inner surface of the micelles. The activities of native HRP in the system of reversed micelles are an order of magnitude higher than those in water solution [26]. The catalytic rate constant for pyrogallol oxidation in water solution is approx. 120 s^{-1} [26], whereas in the reversed micelles the optimum value is 3000 s^{-1} (Figure 1a).

Kinetic data obtained with recombinant non-glycosylated HRP using a number of different electron donors are presented in Figures 1b–1e (in which the donors were pyrogallol, guaiacol, *o*-dianisidine and *o*-phenylenediamine respectively). The profiles obtained for native and recombinant HRP show a number of principal differences.

First, it is clearly seen that the first optimum in Figures 1b–1e is observed at the same degree of hydration of surfactant, independently of the substrate origin. According to our concept and as judged by sedimentation analysis (Table 2) this optimum should be ascribed to the monomeric form of recombinant HRP. At $w_o = 10$ the sedimentation coefficient determined experimentally (22 S) was equal to that calculated theoretically for the 34 kDa protein. Surprisingly, the first optimum for recombinant HRP was observed at a lower degree of hydration of surfactant ($w_o = 10$) than that calculated from the protein molecular mass (34 kDa; $w_o = 12.5$). This indicates that recombinant HRP is tightly folded owing to artificial reactivation from *E. coli* inclusion bodies. We have recently demonstrated that the substrate specificity of recombinant HRP differs from that of native HRP: the recombinant enzyme has a higher activity towards 2,2′-azino-bis(3-ethyl-benzothiasoline-6-sulphonate) and ferrocyanide (2000–4000 s^{-1}) and lower activities towards phenolic substrates (200 s^{-1}) [34,35]. It has been proposed on the basis of site-directed mutagenesis studies that phenolic substrates are able to penetrate into the enzyme active site [36,37] and thus the changes in the substrate specificity of recombinant HRP provide indirect evidence for its tight folding. This is consistent with the conclusions that we made earlier on the basis of irradiation studies on native and recombinant HRP [34,35]. Recombinant HRP exhibits an initial decrease in catalytic activity and then is comparatively stable over a wide range of irradiation doses, in contrast with native HRP. The substrate specificity of this comparatively stable conformation is altered compared with the initial one and becomes close to that of the native conformation [35]. This observation was interpreted in terms of a tightly folded molecule of recombinant HRP that easily forms a more stable but less tight conformation under conditions of irradiation [35].

The micellar matrix enhances the differences in specific activities of native and recombinant enzymes even more because of the activation effect on the native glycosylated enzyme. No activation effects were observed for recombinant HRP: the catalytic rate constants for the oxidation of pyrogallol, guaiacol, *o*-dianisidine and *o*-phenylenediamine in the reversed micelles (Figures 1b–1e) were in the same range as those in water solution (60, 140, 200 and 100 s^{-1} [35]).

Secondly, the dependence of catalytic activity of recombinant HRP on the degree of hydration of the surfactant has two maxima instead of the single maximum observed for native HRP. This type of dependence is characteristic of oligomeric enzymes capable of dissociating into subunits and/or forming supramolecular aggregates of various compositions. In this particular case it reflects the ability of recombinant HRP to form catalytically active dimers as judged by sedimentation analysis (Table 2). At $w_o = 20$ the sedimentation coefficients measured for two fractions were 24 and 29 S, which is close to the theoretical values calculated for the proteins of 34 and 68 kDa, namely 25 and 31 S. The equilibrium between monomeric and dimeric HRP in the reversed micelles was affected by the peroxidase substrates (Table 2). The substrates with a more complex structure lowered the concentration of dimers, whereas smaller substrates increased it. Peroxidase substrates are known to form enzyme–substrate complexes and thus the decreased concentration of dimeric HRP in the presence of *o*-dianisidine and pyrogallol provides indirect evidence for substrate binding at the interface between monomeric HRPs.

Thus the system of reversed micelles demonstrates for the first time the tendency of the non-glycosylated recombinant HRP to form dimeric structures. This indicates that the formation of dimeric structures by haem-containing peroxidases does not occur by chance but could be encoded genetically.

Thirdly, attention should be paid to the catalytic activity of dimeric recombinant HRP relative to the monomeric enzyme. According to the results presented in Figures 1b–1e the catalytic activity profiles for recombinant HRP depend on the substrate's origin. For pyrogallol (Figure 1b) and guaiacol (Figure 1c) the activity profiles are similar. The catalytic activity of monomeric recombinant HRP towards guaiacol is slightly higher than that for the dimeric form (Figure 1c); the reverse is true when pyrogallol is used as a substrate (Figure 1b). The picture observed for two other substrates is excitingly different: dimeric recombinant HRP is much more active towards *o*-phenylene diamine

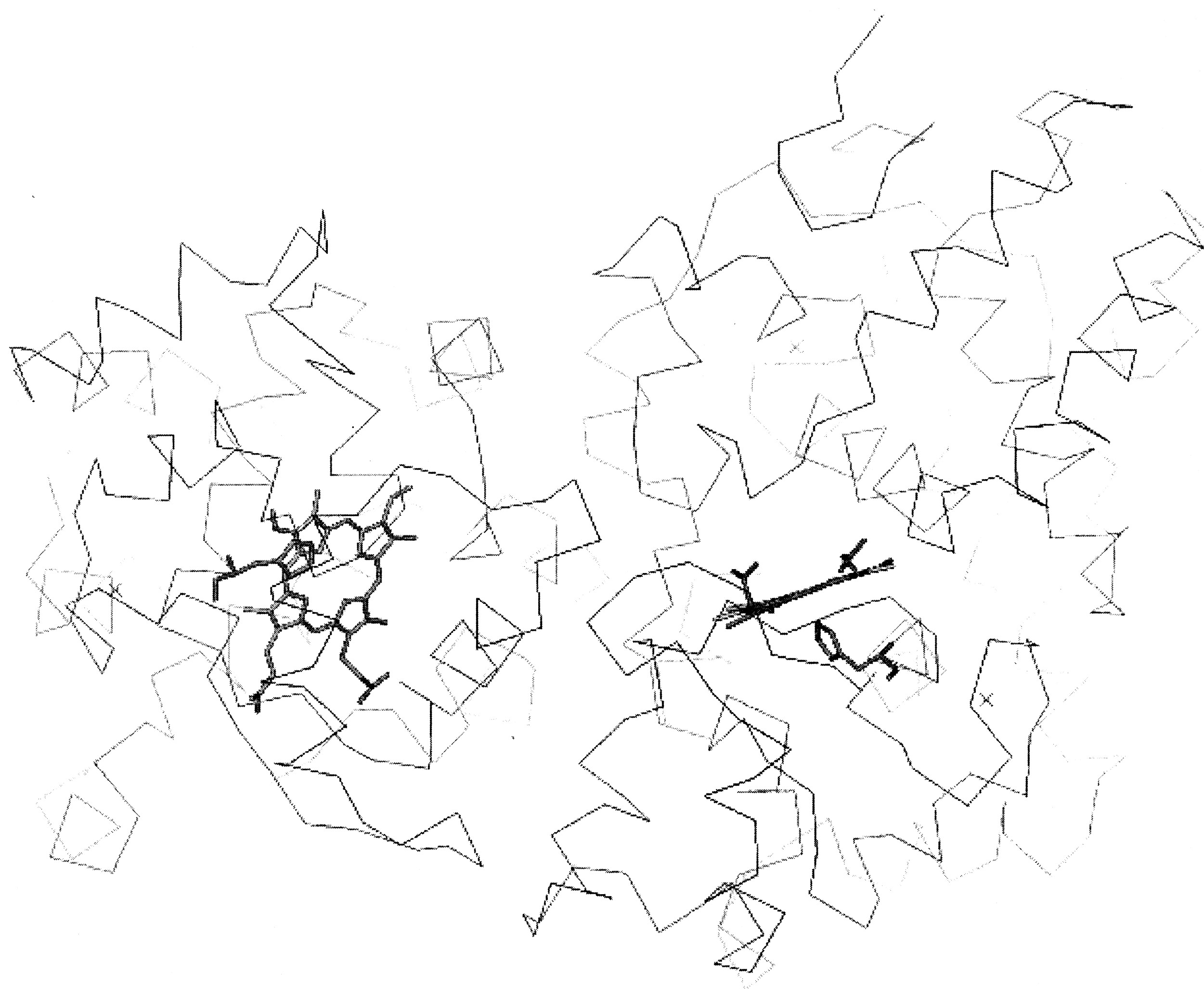

Figure 2 Model structure of the recombinant HRP dimer (3) with the screened entrance to the haem-binding pocket

The left enzyme molecule is shown from the proximal side and the right molecule from the haem propionic acids site.

than monomeric (Figure 1e), and is nearly inactive towards *o*-dianisidine (Figure 1d). Taking into account the ratio of monomeric and dimeric HRPs (Table 2) in the presence of a particular substrate and assuming the catalytic activity of monomeric HRP in the micelles with $w_o = 10$ to be equal to that in micelles with $w_o = 20$, one can easily calculate that the activity of dimeric HRP is 1.3-fold higher towards guaiacol, 1.5-fold higher towards pyrogallol and 2-fold higher towards *o*-phenylenediamine compared with the monomeric enzyme, and that dimeric HRP exhibits no activity towards *o*-dianisidine.

Fourthly, the degree of hydration corresponding to the second activity maximum is shifted slightly ($w_o = 18$ for pyrogallol, $w_o = 20$ for guaiacol, $w_o = 23$ for *o*-phenylenediamine), whereas the first maximum is observed at the same degree of hydration ($w_o = 10$) independently of the substrate origin. Thus there should be a number of closely related conformations of HRP dimeric structures induced by the micellar matrix that differ in their reactivities towards various substrates depending on the mode of active-site screening. Such an assumption, i.e. the existence of a number of dimeric structures with close energies, seems logically relevant, especially if one remembers that energy minimization for the cytochrome *c* peroxidase–cytochrome *c* complex yielded a structure completely different from that obtained from X-ray crystallography [38]. Detailed thermodynamic calculations have shown that manipulations with cytochrome *c* arrangements on the enzyme surface gave a number of equally probable locations.

The experimental results were used as a basis for structural modelling of the recombinant HRP dimer. The analysis of the dimeric structure of pea ascorbate peroxidase [3] showed that the monomers make contact at the side opposite to the active site in the region of α-helices A and D. The main role is played by ionogenic groups forming four ionic bonds: Arg^{24}(A)-Glu^{112}(B), Arg^{24}(B)-Glu^{112}(A), Lys^{18}(A)-Asp^{229}(B) and Lys^{18}(B)-Asp^{229}(A). In HRP the structural analogy allowed us initially to model two possible dimers (structures 1 and 2 not shown), each with two ionic bonds at the site of the contact: (1) Glu^{279}(A)-Arg^{118}(B), Glu^{279}(B)-Arg^{118}(A) and (2) Asp^{20}(A)-Lys^{232}(B), Asp^{20}(B)-Lys^{232}(A). Energy minimization demonstrated that these structures could be formed in principle and gave the following ellipsoid radius sizes $40 Å \times 27 Å \times 27 Å$ (1) and $34 Å \times 22 Å \times 22 Å$ (2) respectively. The formation of dimer (2)

was more advantageous (10 kcal/mol). One of the glycosylation sites, residues 214–216, was screened in the dimeric structures. However, in both cases the surface of the contact was rather far from the active site and did not screen the entrance to the haem-binding pocket.

Because the position of the second maximum in Figures 1b–1e depends on the substrate origin, we propose that the micellar matrix assists in the formation of a third dimeric structure (3) based on hydrophobic interactions screening the active site of the enzyme. Such a structure, represented in Figure 2, was the most advantageous in terms of energy minimization and showed an energy 40 kcal/mol less than that of (2). Dimer (3), created by means of structural modelling, has an ellipsoid shape (33 Å × 24 Å × 24 Å) and contains three contact regions formed by polar but non-ionic groups screening the entrance to the active site. This structure should be very selective for the substrate origin in terms of active site accessibility, explaining the shifts in the second maximum in the region $w_o = 18$–23 (31–39 Å). Moreover, three of four HRP glycosylation sites, i.e. residues 57–59, 186–188 and 214–216, are screened. Oligosaccharide chains contribute significantly to the molecular mass of native HRP (22 %) and interfere with the crystallization of the native enzyme. The presence of oligosaccharide chains might be the reason for the inability of native HRP to form dimeric structures. Thus the application of molecular mechanics methods is in agreement with the fact of the dimeric recombinant HRP formation discovered in this study.

The experimental results obtained on wild-type recombinant HRP in the system of reversed micelles show for the first time the existence of dimeric structures with an altered substrate specificity, in a non-glycosylated plant peroxidase. The equilibrium between monomeric and dimeric HRP is affected by the peroxidase substrates. In terms of plant physiology it is the first indication of microenvironmental effects altering the catalytic properties of peroxidases and serving the purpose of modulating enzyme substrate specificity *in vivo*.

We thank Dr. David Schuller (Irvine, CA, U.S.A.) and Professor Robert van Huystee (London, Ont., Canada) for the coordinates of the crystal structure of peanut peroxidase. We also thank Gesellschaft für Biotechnologische Forschung mbH (Braunschweig, Germany) for financial support in purchasing the SGI working station and Insight II program package. The work was supported in part by INTAS grant N 93-2223-ext.

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Received 4 April 1997/15 July 1997; accepted 13 August 1997