

---

REVIEW

---

## Horseradish Peroxidase: Modulation of Properties by Chemical Modification of Protein and Heme

G. S. Zakharova<sup>1,2</sup>, I. V. Uporov<sup>3</sup>, and V. I. Tishkov<sup>1,2,3\*</sup>

<sup>1</sup>*Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33,  
119071 Moscow, Russia; E-mail: vitishkov@gmail.com*

<sup>2</sup>*Innovations and High Technologies MSU Ltd., 109559 Moscow, Russia*

<sup>3</sup>*Chemical Faculty, Lomonosov Moscow State University, 119992 Moscow, Russia*

Received June 24, 2011

Revision received July 7, 2011

**Abstract**—Horseradish peroxidase (HRP) is one of the most studied enzymes of the plant peroxidase superfamily. HRP is also widely used in different bioanalytical applications and diagnostic kits. The methods of genetic engineering and protein design are now widely used to study the catalytic mechanism and to improve properties of the enzyme. Here we review the results of another approach to HRP modification—through the chemical modification of amino acids or prosthetic group of the enzyme. Computer models of HRPs with modified hemes are in good agreement with the experimental data.

DOI: 10.1134/S0006297911130037

**Key words:** horseradish peroxidase, HRP, heme, chemical modification, catalytic properties, stability, computer modeling

Peroxidases (EC 1.11.1.X) are enzymes of the oxidoreductase class that catalyze the oxidation of a wide range of substrates using hydrogen peroxide. Their molecular weight ranges from 17 to 84 kDa and the polypeptide chain from 153 to 753 amino acids. The proteins usually include 10–11  $\alpha$ -helices, while  $\beta$ -sheets are absent or rare. Most peroxidases consist of two structural domains with a heme, ferriprotoporphyrin IX prosthetic group, in a hydrophobic pocket (Fig. 1) [1].

The generally accepted classification proposed in 1992 [2] divides peroxidases into two large superfamilies, animal peroxidases and plant peroxidases. The superfamily of plant peroxidases is further divided into three classes. This system initially based on amino acid sequence comparison was later confirmed by X-ray data obtained for representatives of different peroxidase classes.

The first class of plant peroxidases includes intracellular enzymes such as ascorbate peroxidase, yeast

cytochrome *c*, and bacterial catalase-peroxidase. These proteins are not glycosylated and contain neither disulfide bonds nor calcium ions. The second class includes secreted fungal peroxidases. The best-studied representatives of this class are lignin peroxidase and manganese peroxidase involved in lignin oxidation as well as peroxidase from *Coprinus cinereus* (previously known as *Arthromyces ramosus*). These proteins are monomeric glycoproteins with four disulfide bonds and two conserved calcium-binding domains. The third class is represented by secreted plant peroxidases. These enzymes are also monomeric glycoproteins with four disulfide bonds and two calcium-binding sites; however, the arrangement of the disulfide bonds and the protein chain folding differs between peroxidases of the second and third classes.

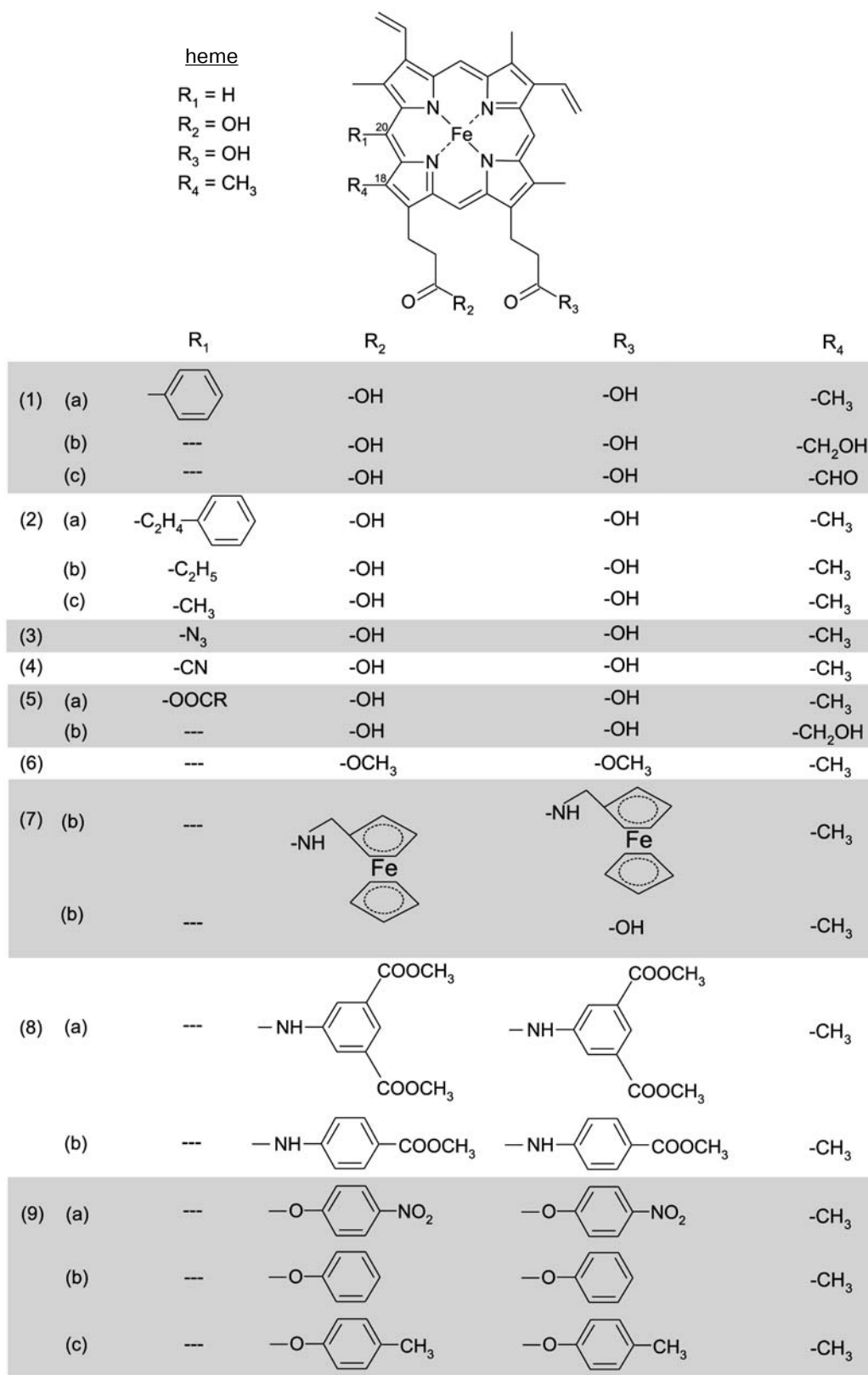
Horseradish peroxidase isozyme C (HRP) is clearly the most popular plant peroxidase. Currently, it is the model enzyme of the third class of plant peroxidases. Although other peroxidases can outperform it in many parameters, HRP finds the widest application in laboratory work. Data obtained over dozens of years allow this enzyme to be used in the development of immune tests [3, 4], various biosensors [5–7], and histological markers [8] as well as in gene therapy [9, 10] and organic synthesis [11, 12].

The studies of the mechanism of HRP action targeted to the improvement or modification of its properties are

---

**Abbreviations:** ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate); BFR, bifunctional reagent; EH, ethylhydrazine; HRP, horseradish peroxidase isozyme C; MFR, monofunctional reagent; MH, methylhydrazine; n-HRP, native HRP; PEH, phenylethylhydrazine; PHZ, phenylhydrazine; rs-HRP, reconstructed HRP; SASA, solvent accessible surface area; SHD, synthetic heme derivative.

\* To whom correspondence should be addressed.



**Fig. 1.** Structure of heme and its chemically modified analogs. Compounds 1-4 are products of the catalytic oxidation of different substrates: 1, phenylhydrazine [87]; 2a, phenylethylhydrazine; 2b, ethylhydrazine; 2c, methylhydrazine [88]; 3, sodium azide [89]; 4, potassium cyanide [90]. Compounds 5a and 5b are products of the incubation in acetate buffer (pH 4.4) or other acids (*n*-caproic and phenylacetic acids) at similar pH [91]. Compounds 6-9 are chemically modified protoporphyrin analogs [97, 98, 101, 102].

still actively performed largely based on directed mutagenesis. This review presents and analyzes the data relying on a different approach, the chemical modification of the enzyme amino acids or the heme porphyrin ring.

### BRIEF DESCRIPTION OF HORSERADISH PEROXIDASE

The roots of horseradish (*Armoracia rusticana*) contain many different peroxidases, but isozyme C has the greatest share among them. The isoelectric point (pI) of HRP is 9, and its activity peaks at pH 6-8. The HRP molecular weight is about 44 kDa, 18-22% of which corresponds to the carbohydrate moiety of the protein. Commonly, eight out of nine asparagine carbohydrate linkage sites (Asn13, 57, 158, 186, 198, 214, 255, and 268) are glycosylated [13]. The carbohydrate moiety composition varies depending on many factors such as growth stage and conditions. Oligosaccharides have a stabilizing effect on the enzyme. They protect the polypeptide chain from proteolysis and modification by free radicals generated in the reaction [14-17].

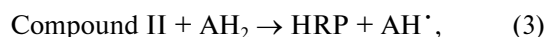
The three-dimensional structure of HRP remained unknown for long, until a recombinant enzyme was produced [18], since all attempts to crystallize the native HRP failed due to its heterogeneous glycosylation. The recombinant unglycosylated enzyme was isolated from *E. coli* cells expressing an HRP gene synthesized on the basis of the amino acid sequence determined by Karen Welinder [19]. To date, the three-dimensional structures of six other plant peroxidases from peanut [20], barley [21], *Arabidopsis thaliana* [22], soybean [23], tobacco (I. G. Gazaryan, personal communication), and royal palm tree [24] have been determined.

The X-ray data demonstrated that the HRP polypeptide chain of 308 amino acids folds into 13  $\alpha$ -helices and three  $\beta$ -sheets [25]. The proper protein conformation is stabilized by four disulfide bonds (Cys11-Cys91, Cys44-Cys49, Cys97-Cys301, and Cys177-Cys209). The protein consists of two domains, distal and proximal, each containing a calcium-binding site where the cations are held by seven coordinate bonds. The structural significance of  $\text{Ca}^{2+}$  has been confirmed by numerous experimental data [26-32] and computer simulations [33]. Since the calcium-binding sites are linked to the active site by a hydrogen-bonding system,  $\text{Ca}^{2+}$  depletion induces significant changes in the enzyme properties [31]. The distal cation has a weaker bond than the proximal one [1]. Its removal roughly halves the catalytic activity and decreases HRP thermostability, stability at extreme pH, and storage stability. The distal  $\text{Ca}^{2+}$  removal induces minor changes on the enzyme three-dimensional structure [28], while complete calcium depletion inactivates the enzyme due to significant conformational changes in the active site [29-31].

The HRP active site contains heme, a prosthetic group that consists of a ferric iron complex with protoporphyrin IX (Fig. 1). The  $\text{Fe}^{3+}$  ion has four coordinate bonds within the heme and one with the nitrogen atom of the imidazole ring in the proximal His170. The sixth coordinate position remains vacant in the relaxed state of the enzyme, but during catalysis, hydrogen peroxide binds to the iron atom to form a six-coordinate complex [34].

HRP can oxidize a wide range of substrates; typical substrates include phenols, indoles, aromatic amines, and sulfonates. The oxidation of aromatic substrates leads to their polymerization, which can be used to purify water from aromatic pollutants [35, 36].

The HRP catalytic cycle includes the following stages:



where  $\text{AH}_2$  is an electron donor substrate and  $\text{AH}^\cdot$  is the radical reaction product.

In the first stage, two electrons are removed from the enzyme (from the iron atom and from the porphyrin ring) as a result of its interaction with hydrogen peroxide. This yields a cation radical called Compound I. Reduction of Compound I by an electron donor to the initial ferri-enzyme proceeds in two stages through the formation of Compound II. Thus, the substrate conversion follows a ping-pong mechanism (Compound I reacts with the first substrate molecule to yield Compound II, which consequently reacts with the second substrate molecule). The catalytic mechanism is considered in more detail in reviews [15, 37-39].

### MUTAGENIC STUDIES OF HORSERADISH PEROXIDASE

This highly efficient approach allows us to identify the amino acids critical for the catalysis, structure maintenance, or stability of the enzyme. It can be used to generate enzymes with improved stability [40, 41] or altered substrate specificity [42]. Such studies underlie practical generation of enzymes with desired properties. In our lab, this approach has been applied to generate biocatalysts with unique properties from formate dehydrogenase and D-amino acid oxidase [43-47].

Amino acid substitutions can be introduced into an enzyme by random (directed evolution, genetic mosaic, etc.) and directed mutagenesis. The latter is often referred to as rational design of proteins considering that point substitutions are introduced after the analysis of enzyme

amino acid sequences or structures. In the case of HRP, rational design is largely used since it is expressed in insoluble form in *E. coli* cells and a refolding from the inclusion bodies is required to make the enzyme active [18]. Frances Arnold and colleagues tried directed evolution to introduce point substitutions into HRP that allow active enzyme expression in *E. coli* cells [48] and methylophilic [49] and baker's yeast [50]. However, the experiments were not successful in *E. coli*, while hyperglycosylation took place in the yeasts: the molecular weight of the carbohydrate moiety could reach 60 kDa, while it is as low as 10 kDa in the natural enzyme.

HRP directed mutagenesis has been applied to elucidate the role of amino acids in heme binding in the distal part of the active site, to study the mechanisms of interaction with  $H_2O_2$  and catalytic substrate oxidation, to identify potential sites for binding different substrate types, and to evaluate the role of calcium cations in the folding and catalytic activity of HRP. The major conclusions of these experiments include: (1) distal His42 and Arg38 play the key role in  $H_2O_2$  binding and heterolytic cleavage of the O—O bond [51–53]; (2) Phe41 is involved in the heme coordination, controls the ferryl oxygen accessibility in Compound I, and modulates the substrate specificity [51, 54–57]; (3) hydrogen bonds between His42, Asn70, and Glu64 connect the distal calcium-binding site and the active site of the enzyme. Apparently, this is required for the proper orientation of the His42 imidazole ring relative to porphyrin [58–60]; (4) the strength of heme binding in the active site depends on the coordinate bond between the heme iron and proximal His170 [61]; (5) the role of Asp247 can consist of making His170 more basic, which maintains the five-coordinate state of heme [25]; (6) Phe179 and Phe142 are critical for binding aromatic substrates [62–64]; (7) Trp117 mediates the energy migration in the molecule and has an effect on the enzyme folding and stability [65, 66]; (8) Thr171 is a factor of the enzyme proper conformation since it connects heme and proximal calcium-binding site [67]; (9) unstructured regions of the polypeptide chain are an important factor of the enzyme stability. Amino acid substitutions in these regions (Thr102Ala, Gln106Arg, Gln107Asp, Thr110Val, and Ile180Phe) decreased the enzyme thermostability [68]; (10) substitutions of Asn13 and Asn268 (glycosylated in native HRP) with Asp increases the enzyme thermostability and resistance to inactivation by hydrogen peroxide [17].

A detailed description of the introduced point mutations and their effects can be found in reviews [15, 37–39].

Thus, genetic engineering tools open up many opportunities to produce an enzyme with new properties; however, mutations are not the only way to modify enzyme properties. Chemical modifications can be equally effective. This approach can be exemplified by the modification of solvent-exposed amino acids.

## CHEMICAL MODIFICATION OF PROTEIN MOIETY

Chemical modification experiments were conducted on both native horseradish peroxidase (n-HRP) with the oligosaccharide moiety and recombinant unglycosylated enzyme. The n-HRP was used in most cases since it allowed the enzyme to be immobilized and conjugated to other proteins (antibodies in particular) through the carbohydrate moiety. In addition, the glycosylated enzyme is more stable and accessible.

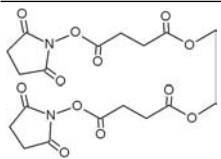
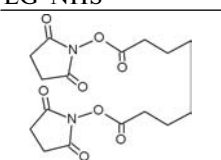
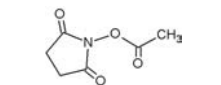
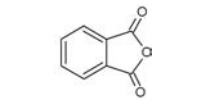
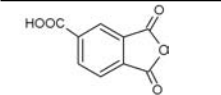
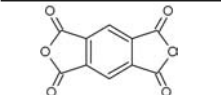
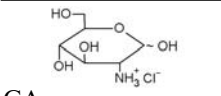
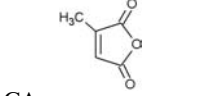
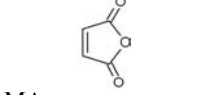
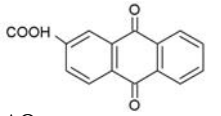
Chemical modification of His [69–71], Tyr, Arg, Asp, and Glu [72] either had no effect or deteriorated the enzyme properties. Only two out of three His residues (40, 42, and 170) proved accessible for the modification, since His170 participates in the heme coordination [71]. The modification of His42 rendered the enzyme inactive; however, it could bind aromatic substrates and form Compound I [70, 71]. These results support the key role of His42 in the energy transfer from the substrate to the prosthetic group.

Positive results were only obtained after lysine residues were modified; accordingly, lysines became the most common target [73–84]. Six lysines in the HRP molecule (Lys65, 84, 149, 174, 232, and 241 [19]) demonstrated different susceptibility to modification. Lys232 proved the most reactive; Lys241 and Lys174 were less reactive; while Lys65, Lys84, and Lys149 are hardly modified [77]. These data agree with other publications demonstrating that there are three modified lysines per HRP molecule [75, 77, 78, 80–84].

Both bifunctional and monofunctional reagents (BFRs and MFRs) were used to modify Lys residues (Table 1). Experimental data indicate that the exposure to BFRs had a stabilizing effect. For instance, the inactivation rate of **EG-NHS**-HRP (hereafter, the left part of the enzyme abbreviation refers to the modification agent; Table 1) at 65°C was four times less than that of the native enzyme [77]. **EG-NHS**-HRP and **SA-NHS**-HRP were also more stable in dimethylformamide (DMF), methanol, and tetrahydrofuran (THF), while the catalytic activity of the modified and native enzymes remained similar.

The modification of HRP by MFRs was also successful. **AA-NHS**-HRP was five times more stable at 65°C and much more active in organic solvents (methanol, DMF, and THF) than n-HRP [75]. The exposure to phthalic anhydride (**PA**) [78–80], glucosamine hydrochloride (**GA**) [78], and anthraquinone-2-carbonic acid (**AQ**) [81, 84] generated not only more stable but also more catalytically active (with high  $k_{cat}/K_m$ ) enzymes in oxidation reactions with phenol and its derivatives (4-amino-, 4-methyl-, and 4-nitrophenol). The **AQ** modification improved both the catalytic ( $k_{cat}$ ) and Michaelis ( $K_m$ ) constants of the enzyme reaction with phenol. In addition, **AQ** proved to be an effective mediator of electron transfer between peroxidase and electrode, which

**Table 1.** Chemical modification of lysine residues in horseradish peroxidase\*

Reagent	Lys	Thermostability		Activity in organic solvents			Catalytic activity		Reference
		t, °C	Δ	solvent	t, °C	Δ	S	Δ	
 EG-NHS	4-5 3 — — — —	65 72.5 — — — —	+ + — — — —	methanol — DMF — THF —	25 60 25 60 25 60	~ + + + + ~	TMB — — — — — —	~ — — — — —	[74, 77]
 SA-NHS	4-5 — — — — —	n.d. — — — — —	n.d. — — — — —	methanol — DMF — THF —	25 60 25 60 25 60	~ + + + + ~	TMB — — — — — —	~ — — — — —	[74]
 AA-NHS	3 — — — — —	65 — — — — —	+ — — — — —	methanol — DMF — THF —	25 60 25 60 25 60	~ + + + + +	TMB — — — — — —	~ — — — — —	[74,75]
 PA	3 2 — — — — —	50 60 — — — — —	+ + — — — — —	methanol DMF — THF — ACN —	60 25 30 60 65 30 60	+ + + + + + +	TMB P AP MP NP — —	~ + + + + + —	[78-80]
 TMA	2	65	~	n.d.	n.d.	n.d.	TMB	—	[79]
 PMA	2	65	~	n.d.	n.d.	n.d.	TMB	—	[79]
 GA	3	50	+	n.d.	n.d.	n.d.	P	+	[78]
 CA	3 — —	50 70 —	+ + —	DMF THF DMSO	25 25 25	+ + +	P — —	~ — —	[82,83]
 MA	3 — —	50 — —	+ — —	DMF THF DMSO	25 25 25	+ + +	n.d. — —	n.d. — —	[82]
 AQ	3 —	50 75	~ +	n.d. —	n.d. —	n.d. —	P —	+ —	[81, 84]

\* Δ, effect (property change relative to unmodified enzyme); S, substrate; **EG-NHS**, ethylene glycol bis(N-hydroxysuccinimidyl succinate); **SA-NHS**, suberic acid bis(N-hydroxysuccinimide ester); **AA-NHS**, acetic acid N-hydroxysuccinimide ester; **PA**, phthalic anhydride; **TMA**, trimellitic anhydride; **PMA**, pyromellitic anhydride; **GA**, glucosamine hydrochloride; **CA**, citraconic anhydride; **MA**, maleic anhydride; **AQ**, anthraquinone-2-carboxylic acid; **DMF**, dimethylformamide; **THF**, tetrahydrofuran; **ACN**, acetonitrile; **DMSO**, dimethylsulfoxide; **TMB**, 3,3',5,5'-tetramethyl benzidine; **P**, phenol; **AP**, 4-aminophenol; **MP**, 4-methylphenol; **NP**, 4-nitrophenol; n.d., no data.

increased the overall efficiency of the process. The catalytic activity of **PA**-HRP was studied both in aquatic solution and in the presence of DMF. In aqueous solution, the enzyme demonstrated a higher catalytic activity ( $k_{\text{cat}}$ ) towards all above-mentioned substrates and a lower  $K_m$  compared to **n**-HRP. In DMF solution, **PA**-HRP  $1/K_m$  was higher (except for 4-nitrophenol), while the catalytic constant was slightly lower. Nevertheless, the efficiency of **PA**-HRP ( $k_{\text{cat}}/K_m$ ) was higher than that of **n**-HRP. The thermostability of **PA**-HRP was also substantially higher: about 80% activity was observed after 90-min incubation at 50°C. The half-life time ( $\tau_{1/2}$ ) of the modified enzyme at 50°C was 10 times that of **n**-HRP (four times at 65°C). The resistance to organic solvents (methanol, DMF, ACN, and THF) also increased.

A similar stability increase was observed in the enzymes modified by citraconic and maleic anhydrides (**CA**-HRP and **MA**-HRP, respectively) [82, 83]. Their stability at room temperature increased in dimethylsulfoxide (DMSO), THF, and DMF. The half-life time of the generated enzymes at 70°C increased roughly three-fold, and their activity after 3-h incubation at 50°C was 82, 74, and 30% of baseline for **MA**-HRP, **CA**-HRP, and **n**-HRP, respectively. Since no significant changes in the thermostability of **TMA**-HRP and **PMA**-HRP were observed, their further analysis was not pursued [79].

Thus, two different approaches to surface chemical modification of HRP based on BFRs and MFRs can be recognized. In the first case, intramolecular cross-links are formed. It was found that there is a single cross-link per HRP molecule in its proximal domain (between Lys232 and Lys241); at the same time, Lys174 is also subject to modification. These results agree with previous data [77] on the accessibility of lysine amino groups for the modification and on their mutual arrangement that delimits cross-links between them by bifunctional agents of definite lengths. In this case, the stabilizing effect can be attributed to an increased rigidity of the molecule around the proximal calcium-binding domain, which can interfere with the dissociation of calcium ion, an important structural factor [77].

The following consequences of HRP modification by MFRs can be recognized: (1) neutralization of the positive lysine residues (e.g. the enzyme acetylation by **AA**-NHS [74]), and (2) Lys charge reversion to negative (e.g. phthalic anhydride modification [78-80]). The reagents mediating electron transfer (such as anthraquinone-2-carbonic acid [81, 84]) should be mentioned specifically, since the main modification target in this case is to increase the efficiency of electron transfer between HRP and electrode rather than to increase the enzyme stability. In terms of enzyme stability improvement, the highest effect was observed when a substituent introduced a single negative charge per modified lysine residue. In the case of the enzyme modification by **TMA** and **PMA**, the number of introduced negative charges per

lysine residue increased to two and three, respectively; however, no stabilizing effect was observed. Some authors believe that changes in the electrostatic interaction pattern of the enzyme can improve its stability [77, 79, 83].

Thus, chemical modification of lysine residues can substantially improve the properties of horseradish peroxidase. However, both mutagenesis and chemical modification of amino acids affect only the protein moiety of the enzyme. An alternative approach is to introduce changes to the heme prosthetic group.

## CHEMICAL MODIFICATION OF PROSTHETIC GROUP

Heme is bound non-covalently in peroxidase, and there are routine methods for heme removal for production of the apoenzyme as well as for reconstruction of the holoenzyme from the apoenzyme and heme [85, 86]. The latter method is actively used in the refolding of recombinant HRP from inclusion bodies [57]. The same techniques were applied to study the impact of porphyrin modification on the properties of the peroxidase.

Two trends can be recognized in the experiments on heme chemical modification: (1) studies on the mechanism of protoporphyrin modifications resulting from its interaction with radical reaction products (autocatalytic modifications) and the impact of these modifications on the enzyme properties; and (2) directed chemical modification of heme to study the properties of HRP with modified protoporphyrin. Figure 1 shows the heme structure and its modification variants studied by different researchers. Hereafter, the numbers of the presented protoporphyrin derivatives are used to abbreviate their complexes with the apoenzyme.

Compounds **1-5** in Fig. 1 result from the autocatalytic heme modification [87-91], and most of them are  $\delta$ -*meso*-heme derivatives (the  $\delta$ -*meso*-carbon in the Fischer nomenclature corresponds to C<sub>20</sub> in the IUPAC system). Introducing substituents into this position usually irreversibly inactivates the enzyme. The reaction with methylhydrazine (MH) is the exception: the enzyme with  $\delta$ -*meso*-methylheme as the prosthetic group (compound **2c**; Fig. 1) purified from the substrates and reaction products by gel filtration gradually restored the catalytic activity, although the rate of the enzyme reactivation was not specified [88].

The heme-modifying radicals formed in the catalytic reaction should also interact with the protein moiety, which can also be a factor of HRP inactivation. Chemical modification of the polypeptide chain has been experimentally confirmed by introducing radioactive labels to the corresponding substrates. The proportion between the modifications in the protoporphyrin and protein moiety proved to substantially depend on the substrate type. For instance, the radicals largely attack the protein moiety

rather than the prosthetic group when phenylhydrazine (PHZ) was used as the substrate [87]. An opposite pattern was observed when phenylethylhydrazine (PEH), ethylhydrazine (EH), methylhydrazine (MH) [88], or azide ion [89] was used as the substrate. In order to separate the effects of protein moiety and prosthetic group modifications on the enzyme inactivation, heme was isolated from modified HRP and incorporated into unmodified apoenzyme. Such reconstructed enzymes proved to have low or no activity [90, 91]. Hence, the heme modification is one of the main factors of HRP inactivation. On the other hand, these data indicate the heme accessibility in the C<sub>18</sub>-C<sub>20</sub> region and the significance of this region for the enzyme activity.

It is of interest to note the following typical features of the autocatalytic modification. In all cases, the rate of enzyme inactivation directly increased with the substrate concentration; however, the highest activity loss was reached at different enzyme/substrate ratios: 11-13 PHZ molecules, 11 PEH molecules, 32 EH molecules, or 80 MH molecules per enzyme molecule. At the azide/enzyme ratio of 60 : 1, the residual activity of the enzyme was 20%. A further increase in the ratio demonstrated a minor improvement. A decrease of the residual activity to 5% of baseline required a 600 molar excess of azide over the enzyme. A complete inactivation of the enzyme by azide (as well as by PHZ) was observed only after its purification by gel chromatography and repeated incubation with azide and hydrogen peroxide. The authors attributed this effect to an unidentified reaction product that binds the enzyme and protects it from the radical attack [87, 89].

Acetate ion is also capable of modifying HRP in the presence of hydrogen peroxide. For instance, the residual activity of HRP was  $5.6 \pm 3.4\%$  after incubating the enzyme in 50 mM acetate buffer (pH 4.4) with a 150 molar excess of H<sub>2</sub>O<sub>2</sub> for 20 min [91]. A more thorough investigation of the process demonstrated that the heme modification was accompanied by its degradation. Direct degradation of intact heme was observed as well. Accordingly, high acetate concentrations and a short-term incubation were used to maximize the yield of the modified enzyme. Similar results were obtained for *n*-caproic or phenylacetic acids instead of acetate, while incubation in citrate buffer yielded no modified protoporphyrin [91]. Thus, heme modification was observed only with alkyl carbonic acids.

It is of importance that heme-containing proteins feature a band in the absorption spectrum around 403 nm (Soret band). The changes in the intensity and position of the Soret band peak reflect the changes in the enzyme structure in the active site region [92]. In all cases of heme autocatalytic modification mentioned above (except acetylation), the activity loss was accompanied by a notable decrease in the Soret band intensity and its shift to the red.

Similar experiments were performed with other heme-containing enzymes and proteins. For instance, reaction with phenylhydrazine inactivated cytochrome P-450 [93], myoglobin [94], and catalase [95, 96]. Catalase was also inactivated by azide ion. However, no heme chemical modification took place in these cases. Instead, the iron atom reacted with the radicals, and the enzyme inhibition resulted from the formation of a stable six-coordinate complex, which prevented its interaction with H<sub>2</sub>O<sub>2</sub>.

Evaluation of the solvent accessible surface area (SASA) demonstrates that nearly all heme in HRP is screened from solvent molecules by the protein moiety except for the C<sub>18</sub>-C<sub>20</sub> region (SASA equals 3.2 and  $\sim 2.4 \text{ \AA}^2$  for C<sub>18</sub> and C<sub>20</sub>, respectively). This indicates that the HRP active site is closed for relatively large molecules, which prevents their interaction with the iron atom.

Thus, these data on autocatalytic modification suggest the following conclusions: (1) HRP heme is embedded in a narrow crevice, which prevents even relatively small molecules to enter in the crevice; (2) the only relatively open protoporphyrin region ( $\delta$ -edge of heme) is subject to radical attack and, hence, modifications; (3) this process inactivates the enzyme.

Let us now consider the second group of heme-modified enzymes generated by the incorporation of synthetic heme derivatives (SHDs) into the apoenzyme. Their structures are shown in Fig. 1 (compounds **6-9**). All these SHDs were produced by chemical modification of heme propionic groups and differ only by the nature of the substituent. Reconstructed HRP (rs-HRP) were obtained using the standard method (with minor modifications, e.g. increased incubation time) [97, 102]. SHD incubation with the apoenzyme led to its incorporation into the protein and restored the HRP catalytic activity. The addition of unmodified heme to the rs-HRP solution did not increase the enzyme activity any more, which confirms the complete SHD incorporation into the enzyme crevice, while similar absorption spectra of rs-HRP and n-HRP confirms the process specificity.

Introduction of a substituent into heme changed both the protein structure and the enzyme properties compared to n-HRP. The molecule was compacted in most cases (excluding **6-rs-HRP** where no notable changes were observed, Table 2; hereafter, the number in the designations of HRP with modified heme corresponds to the number of heme structure in Fig. 1). The compaction can explain the higher thermal stability of **8-** and **9-rs-HRP** than that of n-HRP: the half-life time  $\tau_{1/2}$  increased 2-4 times [97, 98]. In addition to the higher thermostability, the stability of the enzyme in organic solvents increased in some cases. The stability of **8-rs-HRP** in acetonitrile, dimethylsulfoxide, and methanol was much higher than that of n-HRP (this pattern was less pronounced for **8b-rs-HRP**). **9a-**, **9b-**, and **9c-rs-HRP** were more stable in dimethylformamide (DMF) solution at 60°C [98].

Introduction of a substituent into the heme also modified the spectral properties of the enzymes. The fluorescence of the protein is due to Trp, Tyr, and Phe residues. The HRP molecule contains a single tryptophan (Trp117), five tyrosines, and 20 phenylalanines [19]. At the excitation wavelength of 295 nm, tryptophan fluorescence predominates in the fluorescence spectrum of the enzyme [99]. Tryptophan fluorescence in n-HRP peaks at 328 nm. The fluorescence spectrum of HRP depends on excitation energy migration from Trp117 to heme. Hence, changes in the enzyme structure in the heme-binding region that affect the Trp117 distance to or orientation relative to the heme plane as well as changes in the tryptophan environment modify its emission spectrum [100]. Reconstructed enzymes with SHDs demonstrate increased fluorescence intensity and peak shift to the red. This further confirms the modified structure of **rs**-HRPs, which is most likely mediated by an increased heme–Trp distance and increased polarity of the tryptophan environment [97, 98].

In all cases, the catalytic activity of **rs**-HRPs differed from that of n-HRP. For instance, the activity towards guaiacol, thiocyanate, and iodide substantially decreased in the case of **6-rs**-HRP. At the same time, the capacity to bind guaiacol remained similar to that of n-HRP, while the dissociation constant of the complex with thiocyanate decreased 20 times [101]. **7a-rs**-HRP demonstrated no catalytic activity towards 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and ferrocenes. At the same time, **7b-rs**-HRP activity increased towards ferrocenes but decreased towards ABTS threefold compared to n-HRP, while their ability to bind ABTS remained unaltered. The authors attribute the changed substrate specificity to the emergence of a new binding site including ferrocene [102]. Decreased  $K_m$  was observed for **8a**- and **8b-rs**-HRP in the oxidation of phenol and its *para*-derivatives (*p*-hydroxybenzoic acid and *p*-aminophenol) [97], which can be due to the enhanced hydrophobic interactions between aromatic substrates and benzene ring of the substituent in the  $R_2$  position (Fig. 1).

SHDs resulting from the esterification of *para*-derivatives of phenol with carboxyl groups of heme had differ-

ent impacts on the enzyme catalytic properties. **9a-rs**-HRP activity and affinity to phenol increased, while the catalytic properties of **9b**- and **9c-rs**-HRP degraded. These changes are attributed to different donor–acceptor properties of the substituents: electron acceptors (*p*-nitrophenol) decrease the electron density of the porphyrin ring, thus accelerating reduction of Compound II to the native enzyme [98].

Thus, experiments on the production of reconstructed horseradish peroxidase with modified heme suggest the following conclusions: (1) incorporation of SHD into the apoenzyme efficiently proceeds even when bulky substituents are used for the modification of the propionic groups; (2) the produced reconstructed enzymes retain more or less catalytic activity (which can substantially outperform that of n-HRP towards certain substrates); (3) the catalytic properties of HRP can be modulated by variation of substituents introduced into the heme.

#### COMPUTER SIMULATION OF HRP STRUCTURE WITH MODIFIED HEME DERIVATIVES

To understand the mechanism and effects of changes in the properties of peroxidase with modified heme derivatives, we performed computer simulations of the three-dimensional structures of the apoenzyme–modified heme complexes. Below is what we consider the most interesting results. The simulations were generated using the Builder module of the Insight II software package (Accelrys, USA). The starting HRP structure was obtained from PDB:1ATJ. Builder tools were used to add extra chemical groups. The simulation was visually analyzed to identify too close contacts between atoms of introduced substituents and neighboring amino acids. If any, torsion angles of side chains of amino acids were varied to move the atoms apart to an acceptable distance (this procedure was applied to Pro141 in **1a**- and **2a**-HRP and Arg31 in **9a**-, **9b**-, and **9c-rs**-HRP). Then the structure was subjected to 500 steps of energy minimization using the Discover-3 module of Insight II to bring it to equilibrium. Energy minimization is justified when the

**Table 2.** Content of secondary structures in wild-type HRP and HRPs reconstructed from apoenzyme and modified heme

Enzyme	WT	6* [101]	7* [102]		8* [97]		9* [98]		
			a	b	a	b	a	b	c
$\alpha$ -Helices	37	32.4	—	—	55	44	50	41	61
$\beta$ -Sheets	13	17.6	—	—	9	12	10	13	7
Loops	49	50	—	—	36	44	40	45	32

\* The numbers of synthetic heme derivatives correspond to Fig. 1.



initial structural disturbance can be minimized by minor atomic shifts in a local area of the protein. Sometimes the structural disturbance was too high to be eliminated by energy minimization. For instance, the benzene ring in  $R_1$  intersected with Pro141 in **1a**-HRP, and this defect could not be removed in this way. In such cases, stepwise simulation was performed: a methyl group was introduced into  $R_1$ ; the structure was subjected to the minimization; the methyl group was replaced with a benzene ring (in the resulting structure, the close contacts between atoms of benzene ring and amino acids allowed energy minimization); and the structure was subjected to the minimization. Molecular dynamics was used at the next step to bring the protein structure to further equilibrium. The standard molecular dynamics protocol was applied at constant temperature ( $T = 300$  K) in vacuum (two calcium cations and bound water molecules were added to the system). The integration time step was 1 fsec and the total simulation time was 20 psec. The CVFF force field was used in all calculations [103]. In all cases, the most significant structural changes took place within the first 5 psec, which indicates the stability of the resulting structures.

Figure 2 (see color insert) presents the results of the **1a**-HRP simulation. The initial and simulated structures are shown in light and dark colors, respectively. One can see the most pronounced structural changes in the region of amino acids 140 to 143. The introduction of the benzene substituent into the heme brings this group to the place occupied by Pro141 in the native enzyme. The steric stress in the modified enzyme structure was released by a small porphyrin ring turning relative to the Fe–His170 axis and heme sliding deeper into the crevice. Phe68 and Phe142 involved in aromatic substrate binding as well as catalytic His42 and Arg38 critical for hydrogen peroxide degradation were also affected. Experimental data indicate that **1a**-HRP reacted with  $H_2O_2$  to form Compound I, but it was not reduced to Compound II after an electron-donor substrate was added. The computer simulation agrees well with the previous suggestion [87] that the absence of **1a**-HRP catalytic activity towards guaiacol is due to steric restrictions for the heme–substrate contact.

In the case of **2a**-HRP (Fig. 3; see color insert) where the benzene ring is not so rigidly connected to protoporphyrin, the shift of amino acids 140–143 is less pronounced. In addition, the close contact between the introduced substituents and Phe141 can be avoided without heme shift. Otherwise the pattern is similar to the previous case, and the inactivation of the enzyme is also likely due to the substrate block of the active site by the  $\delta$ -*meso*-substituent.

The structure of the complex between the dimethyl ester of protoporphyrin and apoenzyme (**6-rs**-HRP) generated by computer simulation is nearly identical to that of the recombinant enzyme (data not shown). However, spectral analysis indicates structural changes in the heme-binding region: the Soret band in the reconstructed per-

oxidase shifted 6 nm to the long wavelength side and its intensity decreased [101]. In addition, the enzyme demonstrated low activity towards both aromatic substrates (e.g. guaiacol) and small two-electron donors (e.g. iodide ion) [101]. The authors attribute the observed decrease in the catalytic activity to the changed heme position in the enzyme crevice as a result of disturbed hydrogen bonding network between the protein moiety and esterified carboxyl groups. In addition, the neutralization of their negative charges by the modification can decrease the efficiency of hydrogen peroxide reduction, thus, decelerating the formation of Compound I. The kinetic constants were not determined for either catalytic stage in [101], hence further studies are needed to explain the low activity of **6-rs**-HRP.

The introduction of bulky substituents into  $R_2$  and  $R_3$  leads to notable conformational changes in the molecule. The position of amino acids adjacent to the modified propionic groups of heme (such as Arg31, Arg75, Asp174, Gln176, and Leu218) significantly changes. The substituents “push apart” these amino acids to free enough space in the resulting crevice. At the same time, the porphyrin ring position does not substantially change (in the case of **9a-rs**-HRP, heme slides slightly deeper into the globule (Fig. 4a; see color insert); in the case of **9c-rs**-HRP, the porphyrin ring slightly turns around the Fe–His170 axis (Fig. 5; see color insert)). The changes in the position of Phe68 and Phe142 are noticeable; however, these amino acids have high B-factor values, which explain their high mobility in molecular dynamics simulations. The active site region (Arg38, His42, and Phe41) remained largely unchanged. Experimental data on enhanced fluorescence of the tryptophan relative to *n*-HRP indicate more pronounced structural changes in **9c-rs**-HRP than in **9a-rs**-HRP [98]. Indeed, Figs. 4 and 5 demonstrate that the difference in the position of amino acids around Trp117 between the modified and unmodified enzymes is more pronounced in **9c-rs**-HRP in the simulations.

As mentioned above, the methods of genetic engineering and protein design prevail in studying the mechanism of enzyme action and production of biocatalysts with desired properties. However, well-known methods of chemical modification based on the structural data still remain relevant. Sometimes these methods make it possible to produce enzymes with properties that cannot be obtained using genetic engineering. This can be clearly demonstrated by HRP derivatives modified by anthraquinone-2-carboxylic acid, which mediates electron transport from the active site to the electrode. In practice, such products can be used to generate reagentless third-generation biosensors. The computer simulation results for the structure of HRP with chemically modified heme are in a good agreement with the experimental data. Thus, computer simulations can be used not only to explain data but also to design experiments for heme modification.

This work was supported by the Russian Foundation for Basic Research (project No. 10-04-00887a).

## REFERENCES

- Banci, L. (1997) *J. Biotechnol.*, **53**, 253-263.
- Welinder, K. G. (1992) *Curr. Opin. Struct. Biol.*, **2**, 388-393.
- Zhang, S., Zou, J., and Yu, F. (2008) *Talanta*, **76**, 122-127.
- Song, Z., Yuan, R., Chai, Y., Zhuo, Y., Jiang, W., Su, H., Che, X., and Li, J. (2010) *Chem. Commun. (Camb.)*, **46**, 6750-6752.
- Yao, H., and Hu, N. (2010) *J. Phys. Chem. B*, **114**, 3380-3386.
- Shan, D., Li, Q. B., Ding, S. N., Xu, J. Q., Cosnier, S., and Xue, H. G. (2010) *Biosens. Bioelectron.*, **26**, 536-541.
- Korkut, S., Keskinler, B., and Erhan, E. (2008) *Talanta*, **76**, 1147-1152.
- Li, J., Wang, Y., Chiu, S. L., and Cline, H. T. (2010) *Front. Neural Circuits*, **4**, 6.
- Greco, O., Rossiter, S., Kanthou, C., Folkes, L. K., Wardman, P., Tozer, G. M., and Dachs, G. U. (2001) *Mol. Cancer Ther.*, **1**, 151-160.
- Tupper, J., Stratford, M. R., Hill, S., Tozer, G. M., and Dachs, G. U. (2010) *Cancer Gene Ther.*, **17**, 420-428.
- Shogren, R. L., Willett, J. L., and Biswas, A. (2009) *Carbohydr. Polym.*, **75**, 189-191.
- Cruz-Silva, R., Amaro, E., Escamilla, A., Nicho, M. E., Sepulveda-Guzman, S., Arizmendi, L., Romero-Garcia, J., Castillon-Barraza, F. F., and Farias, M. H. (2008) *J. Colloid Interface Sci.*, **328**, 263-269.
- Wuhrer, M., Balog, C. I. A., Koeleman, C. A. M., Deelder, A. M., and Hokke, C. H. (2005) *Biochim. Biophys. Acta*, **1723**, 229-239.
- Clarke, J., and Shannon, L. M. (1976) *Biochim. Biophys. Acta*, **427**, 428-442.
- Veitch, N. C. (2004) *Phytochemistry*, **65**, 249-259.
- Tams, J. W., and Welinder, K. G. (1998) *FEBS Lett.*, **421**, 234-236.
- Asad, S., Khajeh, K., and Ghaemi, N. (2010) *Appl. Biochem. Biotechnol.*, **164**, 454-463.
- Smith, A. T., Santama, N., Dacey, S., Edwards, M., Bray, R. C., Thorneley, R. N. F., and Burke, J. F. (1990) *J. Biol. Chem.*, **265**, 13335-13343.
- Welinder, K. G. (1979) *Eur. J. Biochem.*, **96**, 483-502.
- Schuller, D. J., Ban, N., Huystee, R. B., McPherson, A., and Poulos, T. L. (1996) *Structure*, **4**, 311-321.
- Henriksen, A., Welinder, K. G., and Gajhede, M. (1998) *J. Biol. Chem.*, **273**, 2241-2248.
- Ostergaard, L., Teilum, K., Mirza, O., Mattsson, O., Petersen, M., Welinder, K. G., Mundy, J., Gajhede, M., and Henriksen, A. (2000) *Plant. Mol. Biol.*, **44**, 231-243.
- Henriksen, A., Mirza, O., Indiani, C., Teilum, K., Smulevich, G., Welinder, K. G., and Gajhede, M. (2001) *Protein Sci.*, **10**, 108-115.
- Watanabe, L., de Moura, P. R., Bleicher, L., Nascimento, A. S., Zamorano, L. S., Calvete, J. J., Sanz, L., Perez, A., Bursakov, S., Roig, M. G., Shnyrov, V. L., and Polikarpov, I. (2010) *J. Struct. Biol.*, **169**, 226-242.
- Gajhede, M., Schuller, D. J., Henriksen, A., Smith, A. T., and Poulos, T. L. (1997) *Nat. Struct. Biol.*, **4**, 1032-1038.
- Haschke, R. H., and Friedhoff, J. M. (1978) *Biochem. Biophys. Res. Commun.*, **80**, 1039-1042.
- Ogawa, S., Shiro, Y., and Morishima, I. (1979) *Biochem. Biophys. Res. Commun.*, **90**, 674-678.
- Howes, B. D., Feis, A., Raimondi, L., Indiani, C., and Smulevich, G. (2001) *J. Biol. Chem.*, **276**, 40704-40711.
- Laberge, M., Huang, Q., Schweitzer-Stenner, R., and Fidy, J. (2003) *Biophys. J.*, **84**, 2542-2552.
- Laberge, M., Szigeti, K., and Fidy, J. (2004) *Biopolymers*, **74**, 41-45.
- Szigeti, K., Smeller, L., Osvath, S., Majer, Z., and Fidy, J. (2008) *Biochim. Biophys. Acta*, **1784**, 1965-1974.
- Huang, Q., Laberge, M., Szigeti, K., Fidy, J., and Schweitzer-Stenner, R. (2003) *Biopolymers*, **72**, 241-248.
- Banci, L., Carloni, P., Diaz, A., and Savellini, G. G. (1996) *J. Biol. Inorg. Chem.*, **1**, 264-272.
- Berglund, G. I., Carlsson, G. H., Smith, A. T., Szoke, H., Henriksen, A., and Hajdu, J. (2002) *Nature*, **417**, 463-468.
- Singh, N., and Singh, J. (2002) *Prep. Biochem. Biotechnol.*, **32**, 127-133.
- Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, N., and Bewtra, J. K. (2001) *Water Environ. Res.*, **73**, 165-172.
- Veitch, N. C., and Smith, A. T. (2001) *Adv. Inorg. Chem.*, **51**, 107-162.
- Ryan, B. J., Carolan, N., and O'Fagain, C. (2006) *Trends Biotechnol.*, **24**, 355-363.
- Gazaryan, I. G., Khushpul'yan, D. M., and Tishkov, V. I. (2006) *Uspekhi Biol. Khim.*, **46**, 303-322.
- Rojkova, A. M., Galkin, A. G., Kulakova, L. B., Serov, A. E., Savitsky, P. A., Fedorchuk, V. V., and Tishkov, V. I. (1999) *FEBS Lett.*, **445**, 183-188.
- Tishkov, V. I., Galkin, A. G., Marchenko, G. N., Egorova, O. A., Sheluho, D. V., Kulakova, L. B., Dementieva, L. A., and Egorov, A. M. (1993) *Biochem. Biophys. Res. Commun.*, **192**, 976-981.
- Serov, A. E., Popova, A. S., Fedorchuk, V. V., and Tishkov, V. I. (2002) *Biochem. J.*, **367**, 841-847.
- Tishkov, V. I., Galkin, A. G., Fedorchuk, V. V., Savitsky, P. A., Rojkova, A. M., Gieren, H., and Kula, M. R. (1999) *Biotechnol. Bioeng.*, **64**, 187-193.
- Tishkov, V. I., and Popov, V. O. (2004) *Biochemistry (Moscow)*, **69**, 1252-1267.
- Tishkov, V. I., and Khoronenkova, S. V. (2005) *Biochemistry (Moscow)*, **70**, 40-54.
- Tishkov, V. I., and Popov, V. O. (2006) *Biomol. Eng.*, **23**, 89-110.
- Tishkov, V. I., Savin, S. S., and Khoronenkova, S. V. (2008) *Russ. Chem. Bull.*, **57**, 1033-1041.
- Lin, Z., Thorsen, T., and Arnold, F. H. (1999) *Biotechnol. Prog.*, **15**, 467-471.
- Morawski, B., Lin, Z., Cirino, P., Joo, H., Bandara, G., and Arnold, F. H. (2000) *Protein Eng.*, **13**, 377-384.
- Morawski, B., Quan, S., and Arnold, F. H. (2001) *Biotechnol. Bioeng.*, **76**, 99-107.
- Smulevich, G., Paoli, M., Burke, J. F., Sanders, S. A., Thorneley, R. N. F., and Smith, A. T. (1994) *Biochemistry*, **33**, 7398-7407.
- Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996) *J. Biol. Chem.*, **271**, 4023-4030.
- Newmyer, S. L., and deMontellano, P. R. O. (1996) *J. Biol. Chem.*, **271**, 14891-14896.

54. Smith, A. T., Sanders, S. A., Thorneley, R. N., Burke, J. F., and Bray, R. R. (1992) *Eur. J. Biochem.*, **207**, 507-519.
55. Veitch, N. C., Williams, R. J., Bray, R. C., Burke, J. F., Sanders, S. A., Thorneley, R. N., and Smith, A. T. (1992) *Eur. J. Biochem.*, **207**, 521-531.
56. Heering, H. A., Smith, A. T., and Smulevich, G. (2002) *Biochem. J.*, **363**, 571-579.
57. Gazaryan, I. G., Doseeva, V. V., Galkin, A. G., and Tishkov, V. I. (1994) *FEBS Lett.*, **354**, 248-250.
58. Tanaka, M., Nagano, S., Ishimori, K., and Morishima, I. (1997) *Biochemistry*, **36**, 9791-9798.
59. Nagano, S., Tanaka, M., Watanabe, Y., and Morishima, I. (1995) *Biochem. Biophys. Res. Commun.*, **207**, 417-423.
60. Nagano, S., Tanaka, M., Ishimori, K., Watanabe, Y., and Morishima, I. (1996) *Biochemistry*, **35**, 14251-14258.
61. Newmyer, S. L., Sun, J., Loehr, T. M., and deMontellano, P. R. O. (1996) *Biochemistry*, **35**, 12788-12795.
62. Veitch, N. C., Gao, Y., Smith, A. T., and White, C. G. (1997) *Biochemistry*, **36**, 14751-14761.
63. Veitch, N. C., Williams, R. J. P., Bone, N. M., Burke, J. F., and Smith, A. T. (1995) *Eur. J. Biochem.*, **233**, 650-658.
64. Howes, B. D., Heering, H. A., Roberts, T. O., Schneider-Belhadadd, F., Smith, A. T., and Smulevich, G. (2001) *Biopolymers*, **62**, 261-267.
65. Gazaryan, I. G., Chubar, T. A., Ignatenko, O. V., Mareeva, E. A., Orlova, M. A., Kapeliuch, Y. L., Savitsky, P. A., Rojkova, A. M., and Tishkov, V. I. (1999) *Biochem. Biophys. Res. Commun.*, **262**, 297-301.
66. Ignatenko, O. V., Gazaryan, I. G., Mareeva, E. A., Chubar, T. A., Fechina, V. A., Savitsky, P. A., Rojkova, A. M., and Tishkov, V. I. (2000) *Biochemistry (Moscow)*, **65**, 583-587.
67. Howes, B. D., Brissett, N. C., Doyle, W. A., Smith, A. T., and Smulevich, G. (2005) *FEBS J.*, **272**, 5514-5521.
68. Ryan, B. J., O'Connell, M. J., and O'Fagain, C. (2008) *Biochimie*, **90**, 1389-1396.
69. Urrutigoity, M., Baboulene, M., and Lattes, A. (1991) *Bioorg. Chem.*, **19**, 66-76.
70. Urrutigoity, M., Baboulene, M., Lattes, A., Soupe, J., and Seris, J. L. (1991) *Biochim. Biophys. Acta*, **1079**, 209-213.
71. Bhattacharyya, D. K., Bandyopadhyay, U., and Banerjee, R. K. (1993) *J. Biol. Chem.*, **268**, 22292-22298.
72. O'Brien, A. M. (1997) *Chemical Modification and Characterization of Horseradish Peroxidase and Its Derivatives for Environmental Applications*: PhD thesis, Dublin City University, Ireland.
73. Ryan, O., Smyth, M. R., and O'Fagain, C. O. (1994) *Enzym. Microb. Technol.*, **16**, 501-505.
74. Miland, E., Smyth, M. R., and O'Fagain, C. (1996) *Enzym. Microb. Technol.*, **19**, 63-67.
75. Miland, E., Smyth, M. R., and O'Fagain, C. (1996) *Enzym. Microb. Technol.*, **19**, 242-249.
76. Garcia, D., Ortega, F., and Marty, J. L. (1998) *Biotechnol. Appl. Biochem.*, **27**, 49-54.
77. O'Brien, A. M., O'Fagain, C., Nielsen, P. F., and Welinder, K. G. (2001) *Biotechnol. Bioeng.*, **76**, 277-284.
78. Liu, J. Z., Song, H. Y., Weng, L. P., and Ji, L. N. (2002) *J. Mol. Catal. B: Enzym.*, **18**, 225-232.
79. O'Brien, A. M., Smith, A. T., and O'Fagain, C. (2003) *Biotechnol. Bioeng.*, **81**, 233-240.
80. Song, H. Y., Yao, J. H., Liu, J. Z., Zhou, S. J., Xiong, Y. H., and Ji, L. N. (2005) *Enzym. Microb. Technol.*, **36**, 605-611.
81. Mogharrab, N., and Ghourchian, H. (2005) *Electrochem. Commun.*, **7**, 466-471.
82. Liu, J. Z., Wang, T. L., Huang, M. T., Song, H. Y., Weng, L. P., and Ji, L. N. (2006) *Protein Eng. Des. Sel.*, **19**, 169-173.
83. Hassani, L., Ranjbar, B., Khajeh, K., Naderi-Manesh, H., Naderi-Manesh, M., and Sadeghi, M. (2006) *Enzym. Microb. Technol.*, **38**, 118-125.
84. Mogharrab, N., Ghourchian, H., and Amininasab, M. (2007) *Biophys. J.*, **92**, 1192-1203.
85. Teale, F. W. J. (1959) *Biochim. Biophys. Acta*, **35**, 543.
86. Torres, E., Baeza, A., and Vazquez-Duhalt, B. (2002) *J. Mol. Catal. B: Enzym.*, **19/20**, 437-441.
87. Ator, M. A., and deMontellano, P. R. O. (1987) *J. Biol. Chem.*, **262**, 1542-1551.
88. Ator, M. A., David, S. K., and deMontellano, P. R. O. (1987) *J. Biol. Chem.*, **262**, 14954-14960.
89. DeMontellano, P. R. O., David, S. K., Ator, M. A., and Tew, D. (1988) *Biochemistry*, **27**, 5470-5476.
90. Chen, Y. R., Deterding, L. J., Tomer, K. B., and Mason, R. P. (2000) *Biochemistry*, **39**, 4415-4422.
91. Huang, L. S., Colas, C., and deMontellano, P. R. O. (2004) *JACS*, **126**, 12865-12873.
92. Strickla, E. H. (1968) *Biochim. Biophys. Acta*, **151**, 70.
93. Jonen, H. G., Werringloer, J., Prough, R. A., and Estabrook, R. W. (1982) *J. Biol. Chem.*, **257**, 4404-4411.
94. Ringe, D., Petsko, G. A., Kerr, D. E., and deMontellano, P. R. O. (1984) *Biochemistry*, **23**, 2-4.
95. Deissero, A., and Dounce, A. L. (1970) *Physiol. Rev.*, **50**, 319.
96. Demontellano, P. R. O., and Kerr, D. E. (1983) *J. Biol. Chem.*, **258**, 558-563.
97. Feng, J. Y., Liu, J. Z., and Ji, L. N. (2008) *Biochimie*, **90**, 1337-1346.
98. Song, H. Y., Liu, J. Z., Weng, L. P., and Ji, L. N. (2009) *J. Mol. Catal. B: Enzym.*, **57**, 48-54.
99. Ugarova, N. N., Savitski, A. P., and Berezin, I. V. (1981) *Biochim. Biophys. Acta*, **662**, 210-219.
100. Chattopadhyay, K., and Mazumdar, S. (2000) *Biochemistry*, **39**, 263-270.
101. Adak, S., and Banerjee, R. K. (1998) *Biochem. J.*, **334**, 51-56.
102. Ryabov, A. D., Goral, V. N., Gorton, L., and Csoregi, E. (1999) *Chem. Eur. J.*, **5**, 961-967.
103. Dauber-Osguthorpe, P., Roberts, V. A., Osguthorpe, D. J., Wolff, J., Genest, M., and Hagler, A. T. (1988) *Proteins: Struct. Func. Gen.*, **4**, 31-47.