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Conformational Differences between Native and Recombinant Horseradish Peroxidase Revealed by Tritium Planigraphy

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Abstract—Significant conformational differences between native and recombinant horseradish peroxidase have been shown by tritium planigraphy, which includes a method of thermal activation of tritium followed by amino acid analysis of the protein preparation. Comparison of radioactivity distribution among the amino acid residues with the theoretical (calculated) accessibility shows that the recombinant enzyme is characterized by high hydrophobicity and compactness of folding. The protective role of oligosaccharides in native enzyme has been confirmed. An unexpected result of the study is a finding on high accessibility of a catalytic histidine residue in solution. An effect of low dose (3 Gy) of irradiation on the accessibility of amino acid residues has been unequivocally demonstrated. The data can be interpreted as swelling of the compact folding and increase in the surface hydrophilicity of the recombinant enzyme. In the case of native enzyme, irradiation does not cause remarkable changes in the accessibility of amino acid residues indicating the possible extensive radical modification of the native enzyme in the life-course of the cell. The catalytic histidine is an exception. It becomes inaccessible after the enzyme irradiation, while its accessibility in the recombinant enzyme increases. An additional observation of a 5-fold decrease in the rate constant towards hydrogen peroxide points to the destructive effect of irradiation on the hydrogen bond network in the distal domain of the native enzyme molecule and partial collapse of the active site pocket.

Key words: native horseradish peroxidase, recombinant horseradish peroxidase, γ-irradiation, labeled compounds, tritium, tritium activation

Horseradish peroxidase (HRP) is one of the enzymes most widely used for applied purposes. The enzyme catalyzes the oxidation of a number of natural and artificial electron donors of organic and inorganic origin by hydrogen peroxide. Many attempts to crystallize native horseradish peroxidase failed because of heterogeneity of enzyme glycosylation. Only the production of recombinant enzyme resulted in successful crystallization [1]. The recombinant enzyme was produced in *E. coli* in the form of inclusion bodies, and to reactivate it a refolding procedure under semi-denaturing conditions (2 M urea) was developed [2, 3]. The recombinant enzyme is deglycosylated in contrast to the native one possessing eight oligosaccharide chains. Nevertheless, the solution of its crystal structure took a couple of years due to the heterogeneity in the crystal structure, i.e., each cell contained from 6 to 8 protein molecules [1, 4].

The catalytic mechanism and role of individual amino acid residues can be elucidated by means of site-directed mutagenesis and subsequent studies of kinetic properties of the mutants produced. This approach leads to a question of the correctness of the assumption of unchanged folding of the mutant protein under artificial conditions. To make a conclusion concerning the physiological significance of enzyme properties, the key question is the conformational identity of native and recombinant enzymes, at least in the active site region.

Indirect indications of differences in active site conformations of native and recombinant horseradish perox-

Abbreviations: ABTS) ammonium or sodium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate); IPTG) isopropyl-β-D-thiogalactopyranoside; HRP) horseradish peroxidase; NHRP and RHRP) native and recombinant horseradish peroxidase, respectively.

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idase were obtained in this laboratory earlier in comparative studies of catalytic properties of the enzymes [5-7]. The comparison of specific activities towards different donor substrates showed a higher activity of recombinant enzyme towards 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and ferrocyanide and a lesser activity towards phenolic substrates and iodide [5]. Since ABTS oxidation is known to occur not at the active site directly, the compactness of the enzyme folding providing good electronic contact through the enzyme molecule becomes principal for its efficient oxidation. Thus, the observed increase in the activity towards ABTS for the recombinant enzyme indicates a more compact folding of the molecule and likely a higher hydrophobicity compared to the native enzyme.

The absence of oligosaccharide chains in the recombinant peroxidase resulted in a 2-3-fold decreased thermal and operational (in the reaction course) stability compared to the native enzyme [6]. The data on radiation-induced inactivation demonstrated the difference in both stability and organization of substrate-binding sites for native and recombinant enzyme [7]. A particular property of the recombinant enzyme was the appearance of a plateau in the dose range of 1-3 Gy, i.e., under arbitrary "low" dose irradiation *in vitro*. The activity plateau was interpreted as a formation of a irradiation-stable intermediate of a "molten globule" type whose conformation is sufficiently flexible to compensate for the radiation-induced damage. The profile of activities towards different substrates for this inactivation intermediate was analogical to that for the native enzyme. This allowed us to suggest that native peroxidase is partially inactivated in the reaction course *in vivo* and the mechanism of this inactivation is close to that of radiation-induced inactivation, i.e., the inactivation occurs via a radical mechanism [7].

Thus, the comparative studies of catalytic properties of native and recombinant enzymes demonstrated some distinct differences that must have a structural basis. Therefore, in the present work, the comparative characterization of accessible surface of native and recombinant enzymes was performed using the method of tritium planigraphy.

This method (see [8] and references therein) is a direct experimental approach evaluating the accessible surface of an object and revealing exposed and hidden amino acid residues in a protein molecule. Modern tritium planigraphy is applicable for investigation of bioparticles of both known [9, 10] and unknown structures [8]. The radioactive probe used in the method, e.g., the atom of radioactive hydrogen isotope tritium, substitutes for hydrogen in C–H bonds of a macromolecule. It is assumed that this substitution has no effect on protein structure and properties. Labeling takes place by a single collision of tritium atoms with the target surface (at 77 K), and the label distribution among amino acid residues reflects their accessibility in the macromolecule.

The present study demonstrates the applicability of the method for a comparative structural characterization of preparations of native and recombinant proteins. For the first time, conformational differences between native and recombinant horseradish peroxidases at the level of the active site conformation and at the molecular level have been shown by a direct method independent of enzyme catalytic properties.

MATERIALS AND METHODS

Reagents. Ammonium 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate) (ABTS), ammonium acetate, hydrogen peroxide, calcium chloride, magnesium chloride, isopropyl-β-D-thiogalactopyranoside (IPTG), sodium dodecyl sulfate (SDS), tris(hydroxymethyl) aminomethane (Tris), oxidized glutathione, dithiothreitol (DTT), and hemin were from Sigma (USA). Bactotryptone and yeast extract were from Difco (USA). Isozyme C of horseradish peroxidase was from Biozyme (UK).

Activity was assayed with 0.4 mM ABTS and 1 mM hydrogen peroxide in 0.1 M Na-acetate buffer, pH 5.0, on a Shimadzu UV 120-02 (Japan) spectrophotometer at 25°C. The extinction coefficient at 405 nm for ABTS oxidation product was taken as 36.8 $mM^{-1}\cdot cm^{-1}$ [11]. Hydrogen peroxide concentration was determined spectrophotometrically (ε_{240} = 43.6 $M^{-1}\cdot cm^{-1}$ [12]).

Protein content was calculated from absorbance according to the formula: c (μg/ml) = $183A_{230} - 75.8A_{260}$ [13], which was shown to give the same values as the method of Lowry [14]. The homogeneity of preparations was tested by SDS-PAGE.

Wild-type recombinant horseradish peroxidase (RHRP) was produced by the protocol developed earlier [3] with some additional modifications as follows. The original plasmid pSA261 with the HRP gene under tac-promoter control was kindly provided by Amersham Biosciences, Inc. (UK). Transformed *E. coli* JM109/pSA261 cells were grown in 1 liter LB-medium with 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 mg/ml ampicillin and 0.4% glycerol at 37°C. Expression was initiated by $2\cdot10^{-4}$ M IPTG in the mid-log phase of cell growth. Biomass was collected and disrupted by sonication (22 kHz, 10 min) in the presence of 2 M NaCl and 10 mM DTT. The mixture was incubated 1.5 h at room temperature, and then the sonication was repeated. The supernatant was removed, and the precipitate was washed with 0.05 M Tris-HCl buffer, pH 8.5, with a subsequent solubilization in 10 ml of 6 M urea containing 1 mM DTT. The solubilized RHRP apoprotein (95% purity, 5 mg/ml) was added drop by drop to 1 liter of the refolding medium containing 2 M urea, 0.7 mM oxidized glutathione, 0.1 mM DTT, 5 mM $CaCl_2$, 5% glycerol in 0.05 M Tris-HCl buffer, pH 9.3, and incubated at 4°C

overnight. Hemin (5 μM) was added after overnight incubation and the mixture was additionally incubated for 24 h. The mixture was supplemented with ammonium sulfate (60% saturation) to precipitate the protein. The precipitate was isolated by centrifugation and dissolved in 30 ml water and then applied in 15-ml portions to a Toyopearl HW 55F column (5.2 × 80 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl. Active fractions were pooled and stored frozen. The typical yield of the active enzyme was 20-25 mg per liter of the refolding medium.

Theoretical calculations of amino acid accessibility for solvent were performed with the Insight II program (Accelrys, USA) by calculating Solvent Accessible Surface Area (SASA) using the 3D structure of RHRP [4] with the Macrodox 3.0 program (University of Technology, Tennessee, USA).

Radiation-induced inactivation was performed with a γ-source with the dose power of $P_\gamma = 0.05$ Gy/sec. Residual catalytic activity was measured 1 h after irradiation termination (incubation at 20°C to insure the termination of all secondary reactions).

Method of tritium thermal activation. The protein target was prepared by spray freezing of the enzyme solution on the reactor wall chilled with liquid nitrogen. Then the reactor was connected to the system and vacuumed. After this, molecular tritium was added to the system to 0.5 Pa pressure. Dissociation of molecular tritium to atomic tritium was performed by a triple pulse of an electric current provided by a tungsten filament inside the reactor with temperatures up to 2000 K. After each 15-sec pulse, the residual gas was sucked out for 1 min. To avoid thermal decomposition of the target and diminish side reactions the reactor was cooled with liquid nitrogen during the labeling. After labeling, the target was vacuumed and melted. The labeled protein was dissolved in a minimum amount of water and dialyzed against a large volume of distilled water at 4°C to remove labile tritium. All steps of labeling were monitored with a Mark-3 liquid scintillation device (Tracor Europa, The Netherlands) using a ZhS8 type cocktail. The efficiency of counting was 50%.

Amino acid analysis of labeled preparations was performed at the Chromatography Branch of the Interfaculty Laboratory of Bioorganic Chemistry of Moscow State University under the supervision of Prof. L. A. Baratova. Acid hydrolysis of the labeled protein was performed with the mixture of 12 M HCl–trifluoroacetic acid (2 : 1 v/v) with addition of 0.001% β-mercaptoethanol in glass ampoules at 155°C for 1 h. The hydrolyzate was 2-fold diluted with water and dried at a SpeedVac Concentrator (Thermo Savant, USA). The preparation was dissolved in 0.1 M HCl before the application to a column. Amino acid analysis was performed at a Hitachi 835 amino acid analyzer (Japan). The resulting specific radioactivities of amino acid residues were calculated by dividing the total radioactivity of the amino acid residue by its total content in accordance with the amino acid analysis data. All analyses were performed in triplicate. The normalized radioactivities were obtained by dividing the radioactivity of individual amino acid residues (peak area) by the total radioactivity (sum of areas of all peaks).

RESULTS AND DISCUSSION

Since traditional enzymology methods and radiation-induced inactivation pointed to significant differences between native and recombinant enzymes, it was interesting to compare the conformations by a method independent of their catalytic characteristics. Tritium is a low energy β-emitter and, because of its short tracking distance, can attack only surface-exposed amino acid residues. Thus, the tritium distribution in individual amino acid residues gives an idea on the accessibility of amino acid residues in a protein. For two identical protein conformations one expects the same distribution of the tritium label among amino acid residues.

Taking into account our previous data on radiation-induced inactivation [7], it was necessary to analyze the effect of low dose radiation (*in vitro*) on the accessible surface of the protein. Therefore, in this work we used tritium planigraphy to analyze both the accessibility of the original protein surface and its changes caused by a low dose of γ-irradiation.

Theoretical calculations of accessible surface of the recombinant enzyme were performed using the enzyme crystal structure coordinates. The accessibility profile (an average of accessibility) of amino acid residues of recombinant horseradish peroxidase is presented in Fig. 1. The highly accessible residues are 16 of 20 Arg, 19 of 26 Asn, 7 of 13 Gln, 10 of 34 Leu, 8 of 17 Pro, and 11 of 23 Thr. The residues of Ala, Tyr, Glu, and Val are less accessible.

The excess of positively charged Arg residues at the surface of peroxidase molecule explains the fact that the isoelectric point of the enzyme is close to pH 9.0. Thus, in the pH range used in this work, the surface of horseradish peroxidase is positively charged. The exposure of hydrophobic resides on the surface may give the recombinant enzyme extra hydrophobicity compared to the native one, whose surface is screened by hydrophilic oligosaccharide chains.

Proline residues known to change the direction of α-helix folding are highly exposed to the surface. The least accessible are histidine residues buried deep inside the heme-binding pocket.

The data on tritium distribution in amino acid residues of native and recombinant peroxidase before and after irradiation with 3 Gy dose of γ-irradiation are presented in Fig. 2. As seen, the profiles of radioactivity of amino acid residues for both native and recombinant

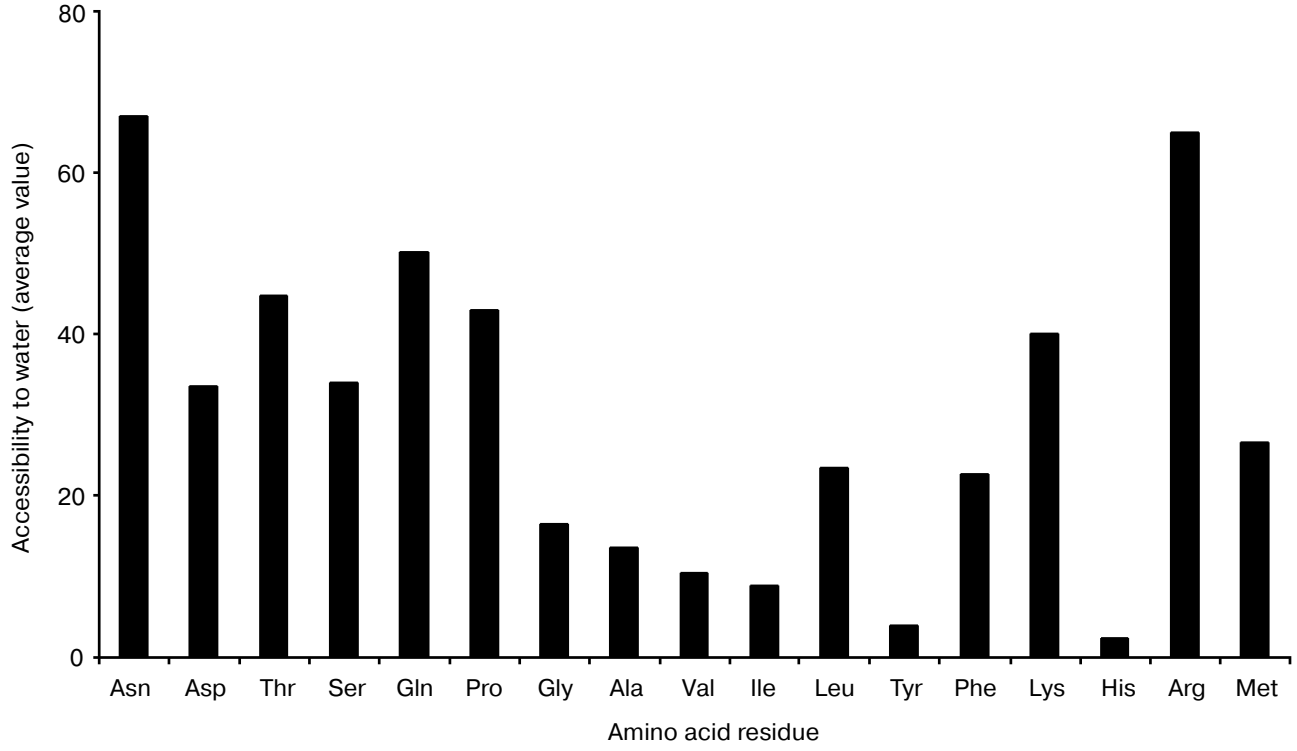

Fig. 1. Profile of average accessibility of amino acid residues of recombinant horseradish peroxidase calculated on the basis of its crystal structure.

enzyme differ from calculated values (see Fig. 1). However, the experimental profile for the recombinant enzyme shows more differences.

Comparison of specific radioactivity of hydrophobic amino acid residues, e.g., Val, Ile, Leu, and Tyr in NHRP and RHRP shows that their accessibility in the recombinant enzyme is much higher than in native. This indicates increased hydrophobicity of the recombinant enzyme surface compared to that calculated from the enzyme crystal structure. The accessibility of almost all amino acid resides in the recombinant enzyme is increased already at 3 Gy dose. Thus, the low dose γ-irradiation easily destroys the compact structure of the recombinant enzyme. The observed increase in the exposure of charged amino acid residues, such as Asn and Asp, can be interpreted as partial unfolding or swelling of a compact hydrophobic structure resulting in an increased exposure of hydrophilic residues.

There is a major difference between the calculated and experimental profiles of amino acid accessibility. This is an extremely high radioactivity of His residues. This suggests that the accessibility of histidine residues (Fig. 2) in the molecule of horseradish peroxidase in solution is much higher than in the crystal. It is important that the active site of peroxidase including all three histidine residues in solution is exposed to the solvent much more than would be supposed. The irradiation of the recombinant enzyme does not result in the changes in His radioactivity, and thus, in its accessibility in RHRP.

Thus, even low doses of irradiation significantly change the conformation of the recombinant enzyme making it more hydrophilic and less compact.

In the case of native enzyme, the most accessible for tritium are Thr, Leu, and Pro residues, which coincides with the theoretically calculated accessibility of these residues. The level of radioactivity of Ile, Val, and Tyr in the native enzyme is much lower than in the recombinant enzyme. The radioactivity of His residues in the native enzyme is also much lower than in the recombinant enzyme, but still much higher than the calculated one. The reason for this could be the lesser exposure of histidines in the native enzyme because of the enzyme glycosylation or significant changes in conformation and possible radical modifications during the life course of the native enzyme *in planta*.

A significant observation is that irradiation results in almost complete loss of accessibility of His residues in the native enzyme. This could originate either from direct modification of the histidines or from partial collapse of the hydrogen bonding network in the distal domain of the enzyme and subsequent conversion of 5-coordinated catalytic His to 6-coordinated. The changes in the rate con-

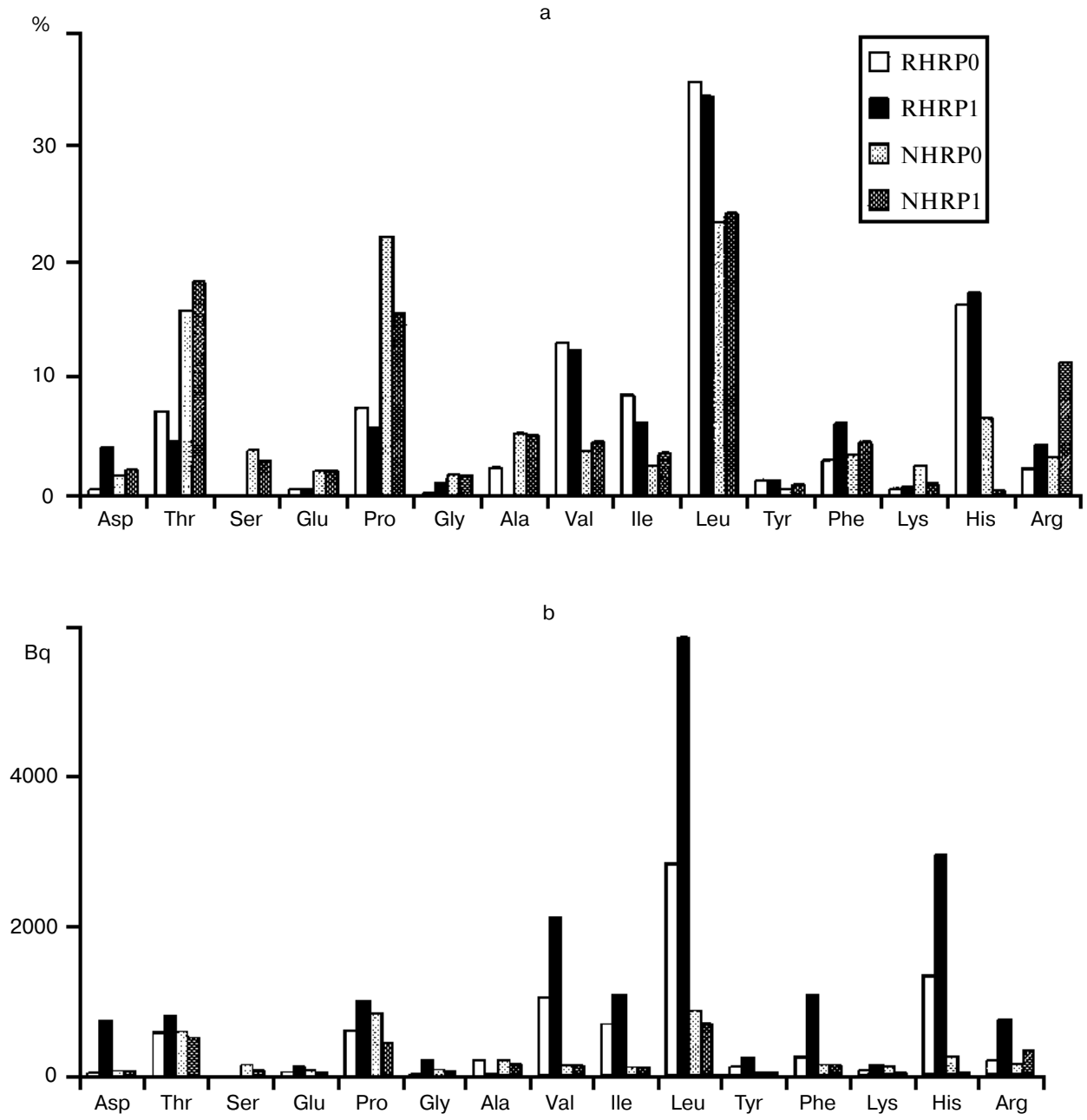

Fig. 2. Tritium distribution in amino acid residues of native (NHRP) and recombinant horseradish peroxidase (RHRP) before and after γ-irradiation in % (a) and towards 1 nmol of protein (b). HRP1) γ-^{3}H; HRP0) ^{3}H.

stants for hydrogen peroxide and ABTS for the native enzyme before and after a low dose irradiation (table) show that the irradiation affects only the rate constant for hydrogen peroxide. The change in this elementary rate constant does not affect the specific activity because this constant is not rate-limiting.

Thus, even a low dose irradiation (3 Gy) resulting in a stable enzyme intermediate is sufficient to change the active site conformation of the native enzyme. The 5-fold drop in the rate constant towards hydrogen peroxide is indirect proof of major changes in the coordination state of the catalytic histidine. The effect of irradiation on His accessibility in the native and recombinant enzyme visualizes major conformational differences between the active sites of the two enzyme forms before irradiation.

Irradiation of native enzyme has almost no effect on accessibility of amino residues except the above mentioned His, and also Pro and Arg. The absence of pro-

Apparent rate constants for ABTS oxidation by hydrogen peroxide catalyzed by native HRP before and after irradiation

Rate constants	Control NHRP	Irradiated NHRP	
		1.5 Gy	3 Gy
k_1, $\mu M^{-1} \cdot sec^{-1}$	12	11	2
k_3, $\mu M^{-1} \cdot sec^{-1}$	1.3	1.2	1.0
k_4, sec^{-1}	3500	5900	2000

nounced changes in accessibility of amino acid residues after irradiation for the native enzyme may point to the fact that the native enzyme, in contrast to the recombinant one, has been subject to radical modification by oxidized forms of phenolic substrates in the course of catalysis *in vivo*. And all possible radical modifications already occurred long before the irradiation experiment. In addition, one cannot ignore the protective effect of oligosaccharide chains exhibiting high radiosensitivity.

The most interesting observation is that irradiation increases the Arg residues accessibility in the recombinant enzyme and even more in the native one at the comparable level of Arg radioactivity in both enzymes before irradiation. Glycosylation sites in the native enzyme are located close to 7 of 16 highly exposed Arg residues; therefore, the lesser accessibility of Arg residues in the native enzyme after irradiation compared to the irradiated recombinant enzyme can be explained by their screening by oligosaccharide chains.

An increase in accessibility of Arg residues after irradiation may reflect also the increased accessibility of lesser accessible Arg residues such as catalytic Arg38, propionate-coordinating Arg31, and located close to the surface Arg298. Low dose of 3 Gy is unlikely to be sufficient to increase accessibility of Arg123 and Arg183 deeply buried inside the globule. A possible increased exposure of catalytic Arg38 participating in binding of hydrogen peroxide and phenolic substrates may have a negative effect on peroxidase catalytic characteristics.

Serine residues in the recombinant enzyme, even after irradiation, were still poorly accessible although their theoretical accessibility in RHRP is rather high. For the native enzyme, the experimental accessibility of Ser residues agrees with the calculated one and remains almost unchanged after irradiation.

In conclusion, the data of tritium planigraphy unequivocally demonstrate the conformational differences between native and recombinant horseradish peroxidase, both at the level of active site and at the molecular level. The recombinant enzyme is compactly folded and highly hydrophobic. Glycosylation of the native enzyme significantly hinders the enzyme surface protecting it from radical attack, in particular, in the region of indicated amino acid residues. The unexpected result of this study is the high accessibility of catalytic histidine in peroxidase molecule in solution, higher than the theoretically calculated values for its accessibility in the crystal.

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