ANALYSIS OF THE ANTIGENIC STRUCTURE OF HOLO- AND APO-FORMS OF HORSERADISH PEROXIDASE

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In order to reveal the conformational changes in holo- and apo-forms of horseradish peroxidase (HRP) the differences in immunogenicity of these forms have been studied by immunochemical techniques. A panel of polyclonal antibodies (PcAb) specific to holo- and apo-HRP was tested by double immunodiffusion (Ouchterlony test) to find the antisera which distinguish the structural changes in peroxidase molecule after heme removal. Two sera specific to apo-HRP were revealed to recognize the epitopes specific only for apo-HRP. Peptide scanning (PEPSCAN) of these PcAb was used to determine linear antigenic determinants for apo-form and compare them with those obtained for holo-HRP earlier. We found most epitopes established for holo-HRP, but some of them were shifted by one-two amino acids. At the same time we revealed several new epitopes specific only for apo-HRP. Some of epitopes placed close to the active site were specific only for holo-HRP. The increased amount of antigenic epitopes established for apo-HRP and partial shifts in their location mean certain conformational changes in peroxidase molecule in a result of heme removal. It also means that apo-HRP is less compact protein with increased flexibility of segments and loops. These facts confirm the role of heme in close approach of two domains and stabilization of enzyme molecule.

Horseradish peroxidase (HRP) is a heme-containing glycoprotein which is one of the most studied member of the plant peroxidase superfamily due to widespread application as a label for immunoassay and DNA probe methods. The most abundant isoenzyme is HRP C with a known amino acid sequence of 308 residues [1]. It has the iron(III) protoporphyrin IX active center, eight neutral carbohydrate chains positioned at Asn residues which stabilize the enzyme towards the action of proteolytic enzymes. The synthetic gene for HRP was expressed in *E. coli* in order to produce the recombinant enzyme and its mutants to study their effects on the catalytic activity [2]. The *E. coli* system produces non-glycozylated recombinant HRP in apo-form, which does not contain heme. The yield of expression is high enough (15–30% of total cell protein), but the protein is obtained in the form of inclusion bodies and the refolding proved to be problematic in view of the complexity of the process. Only low yeilds of enzymatic activity were obtained as a result of the refolding of recombinant HRP *in vitro* [3]. Essential distinction in tertiary structure of holo- and apo-enzyme may be the reason for refolding failure. The structures of both forms of the hemoproteins including HRP were extensively studied by circular dichroism [4, 5], tryptophan fluorescence [5], and NMR [6]. They established certain disturbances in secondary structure of HRP after heme removal. Another feasible way of looking at this problem involves the application of immunological methods based on the ability of immune system to recognize the specified elements of foreign molecule structure by producing the specific antibodies towards these elements. The antibodies are widely used for identification, quantitative analysis and purification of a large number of biologically active compounds due to their distinguishing features such as a variety of specificities and reversibility of binding with antigen. Antibodies were also found to be the probes for the surface structure of the protein. Comparative study of composition of antibodies produced towards different immunogens and localization of corresponding epitopes make possible the determination of structural similarity and distinctions for the proteins used as immunogens.

Antigenic determinants or epitopes present the regions of a protein to which antibodies bind. They have been classified as linear (continious) and discontinious respectively [7]. The linear epitopes are defined as short stretch of the polypeptide chain. Discontinious or conformational consist of residues found at certain distances along the polypeptide chain but brought close to each other in space. Epitope mapping of proteins by means of peptide scanning (PEPSCAN) [8–10] is a new approach to determine the linear antigenic determinants. The method consists in testing of the interaction of specific antibodies with short peptides overlapping the amino acid sequence of the protein. Recently we used this method to determine linear antigenic determinants of holo-HRP [11]. The aim of present study was to investigate the distinction in immunogenicity of holo- and apo-HRP and to compare the linear antigenic determinants of both forms by means of PEPSCAN technique.

Materials and Methods

Horseradish peroxidase (HRP) was obtained from Biozyme (Blaenavon, UK) and was of the highest grade of purity available. Chemicals and substrates for HRP were

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supplied by Sigma (St. Louis MO, USA). Alkaline phosphatase was supplied by Boehringer Manheim (Manheim, Germany). Conjugate of sheep anti-rabbit immunoglobulin (secondary antibody) with alkaline phosphatase was obtained by means of glutaraldehyde followed by the purification on column with Toypearl HW-55. The water used for dilution and preparation of buffers was obtained by distillation.

Solid-phase synthesis of hexapeptides on polyethylene pins was performed as described earlier [11].

96-well polystyrene microtiter plates (high binding) were supplied by Biohit (Helsinki, Finland).

Antisera. Polyclonal antibodies against holo- and apo-peroxidases were produced in rabbits by several subcutaneous (along the vertebral column) injections of 1 mg of the protein in 0.5 ml of PBS mixed with an equal volume of complete Friend's adjuvant. The immunization procedure was repeated six times at two-week intervals. Blood was sampled one week after the last injection (1st cycle).The following injections were made with 1 month intervals, and the blood was collected one week after each injection. Control sera were obtained from the same animals before immunization, one week after injection of 1 ml of PBS. Immunoglobilin-containing fraction was produced from antiserum by precipitation with sodium sulfate followed by centrifugation and gel-filtration on G-25.

Apo-HRP was prepared from the holo-HRP by removing heme with methylethylketone at pH 2.5 and +3 °C accordingly [12]. The aqueous solution containing apo-HRP was neutralized with 1 M ammonium carbonate to pH 7.0 and then purified on a column with sephadex G-25. Then 0.01 M calcium chloride was added up to the final concentration of 0.1 mM, at the final stage the solution was dyalized against 0.05 M K-phosphate buffer pH 8.0 at +3 °C.

Apparatus. Spectrophotometric measurements were performed on a spectrophotometer Schimadzu UV-1602 (Japan). Measurements of optical density for 96-well microtiter plates were performed on a microtiter plate reader (Molecular Devices, Palo Alto, CA, USA).

Enzyme-linked immunosorbent assay (ELISA). The wells of the plate were coated with 100 μl of HRP at a concentration of 4 μg/μl overnight at +4 °C. The wells were washed with PBST (3x300 μl). Different dilutions of holo- or apo-HRP specific antibodies in PBST were added to coated wells, respectively. The plates were incubated for 1 h at 37 °C, then washed with PBST (3×300 μl). 100 μl of secondary antibody-alkaline phosphatase conjugate solution diluted 1/5000 in PBST were added to the wells followed by another 1 h incubation at 37 °C and washing with PBST (4×300 μl). 100 μl fresh prepared substrate solution contained 1 mg/ml *p*-nitrophenylphosphate in 1 mM diethanolamine pH 9.8 adjusted with HCl containing 0.5 mM $MgCl_2$ were added to each well. The optical densities were read at 405 nm. The binding of non-immune rabbit serum was employed as a control.

Double immunodiffusion (Ouchterlony test) [13]. Three wells disposed in the form of triangle were made in agar gel. The solutions of holo-HRP (15 μg/ml) and apo-HRP (30 μg/ml) were injected in two of them, and the antiserum being studied—in the third well. The dilution of antiserum optimal for precipitate formation was determined in separate test. After overnight incubation the form of precipitation lines was analyzed.

Peptide scanning (PEPSCAN). The assay was performed on the pins with bound hexapeptides overlapping the amino acid sequence of HRP as described in [14]. The nonspecific sorption was blocked with a blocking buffer (PBS containing 0.1% Tween 20, 0.1% casein hydrolysate, 0.02% sodium azide) for 1 h at 37 °C. Then the pins were incubated with specific or nonspecific (control) antibodies (175 μl/pin) in a concentration of 5–10 μg/μl in the blocking buffer overnight at +4 °C. After that the pins were washed (4×10 min) with PBST followed by the incubation with peroxidase-labeled secondary antibodies in the blocking buffer without sodium azide for 1 h at 37 °C. The pins were then washed again as described above and incubated in a substrate solution (0.5 mM ABTS, 2 mM H_2O_2 in 0.1 M Na-citrate buffer pH 4.5, 150 μl/pin) for 45 min at RT. The reaction was stopped by removing the pins from the wells of microtiter plate, and the optical density of colored solutions in the wells was then measured at 405 nm (A_{405}). After each assay the pins were washed by sonication in a disruption buffer containing 0.1 M sodium phosphate pH 7.2, 1% sodium dodecyl sulfate, 0.1% mercaptoethanol at 60 °C, then the pins were washed twice with water (60 °C) and at the end with boiling ethanol.

In order to separate signals from noise and specific binding from non specific we tested the binding of non immune rabbit serum (control) with the set of hexapeptides at the same conditions in independent experiment. The optical densities obtained for each of hexapeptide were normalized to the value of optical density for background well obtained in the same experiment. Then the normalized optical densities obtained for control were subtracted from the normalized optical density of antiserum being investidated. Signals greater than mean of modified A_{405} by the value of 2 standard deviations were taken as significant as described in [15].

Results and Discussion

In order to investigate the conformational modifications of peroxidase molecule after removing of heme we prepared apo-peroxidase and used both forms of the enzyme as immunogens to produce specific polyclonal antibodies in rabbits. Apo-HRP was obtained from holo-HRP by means of heme extraction with methyl ethyl ketone at acidic conditions (pH 2.5) accordingly [12]. The apo-enzyme was finally admixed with 5% of holo-enzyme but such negligible amount does not affect the results of ELISA test.

To produce polyclonal antibodies rabbits were immunized with holo- and apo-HRP by similar schemes. Polyclonal antisera obtained from several rabbits after various cycles of immunization (from second to sixth) were compared to their specificity and affinity towards corresponding immunogen by means of indirect ELISA with conjugate of secondary antibodies labeled with alkaline phosphatase. The interaction of non immune rabbit serum at the same conditions was employed as control. The specific antibodies were compared with the values of immunological titer

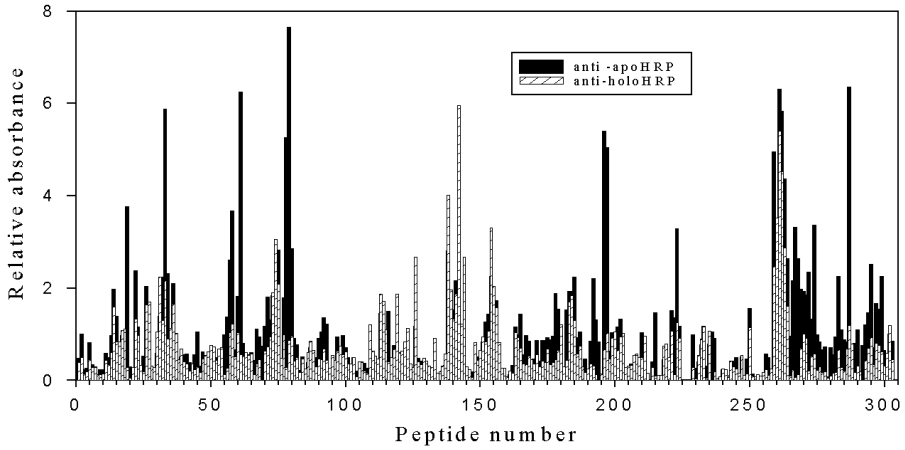

Fig. 1. Interaction of hexapeptides from the sequence of HRPC with antisera towards holo- and apo-HRP.

(dilution corresponds to 50% binding of antibodies with immobilized antigen). 5 antisera towards holo-HRP and 5 antisera towards apo-HRP with maximal values of immunological titer were selected for further investigation

To study the identity of antigens of holo- and apo-HRP the antisera selected were examined by double immunodiffusion in agar gel (Ouchterlony test) accordingly [13]. Three wells disposed in the form of triangle were made in agar gel. The solutions of holo- and apo-HRP were injected in two of them, and the antiserum being studied was injected in the third well. After overnight incubation we analyzed the form of precipitation lines. The formation of a single precipitation symmetric curve was revealed for all antisera towards holo-HRP and most, except two, antisera towards apo-HRP. Such form of precipition line means absolute identity of antigens for holo- and apo-peroxidases recognized by these antisera. At the same time we detected the formation of the non symmetric precipitation line for two antisera specific to apo-HRP (A-2081-2 and A-2081-3), when one of the precipitation lines formed in opposite of the well with apo-HRP was longer than another and it was beyond the intersection of both lines. By this it meant that there antisera recognize certain differences in antigenic structure of both forms of HRP, and there are several antigens only inherent for apo-HRP.

Ouchterlony test is a qualitative method, and it allows only to establish the presence of distinctions between two forms of peroxidase without characterization of pecular antigens. In further investigation we aimed to elucidate which fragments of HRP molecule modify the conformation during the heme removal. To localize the linear antigenic determinants of apo-HRP we used peptide scanning PEPSCAN. For this purpose we tested the interaction of immunoglobulins from the sera selected in Ouchterlony test with the panel of hexapeptides overlapping the amino acid sequence of HRP. The binding of antibodies to the set of hexapeptides can be presented as a characteristic pattern where the abscissa is the number of peptide and the ordinate is the optical density obtained in ELISA that is proportional to the concentration of antibodies bound with that peptide. To compare the patterns obtained for different immune sera we modified the optical densities obtained for each of hexapeptide as described in Materials and Methods.

Figure 1 presents the patterns obtained for anti-apoHRP antiserum and anti-holoHRP antiserum modified in the way described above. First of all, we revealed that the most part of epitopes found earlier as specific for holo-HRP [11] are also peculiar for apo-form. At the same time we detected the increased total amount of epitopes for apo-HRP. Both NH_2-terminal and COOH-terminal regions of apo-HRP were found to become more immunogenic. Several epitopes (19, 61, 78, 196, 215, 266, 275) were detected only for apo-form. Some epitopes revealed for holo-HRP were detected also for apo-form but with certain shift by 1–2 amino acids (19, 75, 266). This is an evidence for partial unfolding of the protein molecule in these regions, and more immunogenic amino acids become more accessible. At the same time we revealed only weal binding of antibodies towards apo-HRP with some of peptides established as epitopes for holo-HRP (138, 142, 126, 154), and some of them include the amino acids which are functionally significant and are involved in the channel through which aromatic substrates gain access to the δ-meso carbon of the heme ring. In general, apo-HRP was established to be more immunogenic when compared with holo-peroxidase.

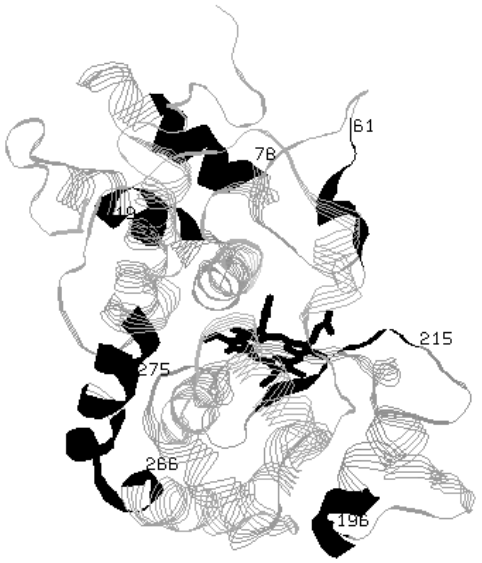

Fig. 2. Schematic representation of epitopes inherent only for apo-peroxidase on the spatial model of HRPC. Regions of the polypeptide chain involved in epitopes are shown as dark solid tapes. The figure also shows the heme.

There are some evidences that antibodies against flexible segments and loops cross-react with short peptides more easily and they are effectively detected with the PEPSCAN method [15]. The increased amount of epitopes and certain shifts in their location when compared with the epitopes for holo-HRP signifies more flexible and less compact structure of apo-peroxidase.

Figure 2 shows the model of the spatial structure of HRPC with the marked regions of the polypeptide chains correspond to epitopes inherent only for apo-HRP. One of them (215) is placed close to the active center, the epitope 275 is placed on the reverse side, the others were found on the surface of the molecule located far enough from the active site. One can conclude that heme removal affects the structure of most of protein globule, and the role of heme consists in close approach of two domains and stabilization of enzyme molecule.

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