NANOTECHNOLOGICAL SIGNAL AMPLIFICATION METHODS FOR IMMUNOASSAY

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Autometallography and enzyme metallography that are methods of enzyme-linked immunosorbent analysis signal amplification are reviewed. The reasons of using them are described. Both methods involve metal deposition onto surface of an already present metal nanoparticle. Therefore both methods are being used in highly sensitive immunoassay using atomic force microscopy as a detection method and in immunohistochemistry. The chemical basis of both methods is given. Numerous examples of their application are reviewed. Some unusual detection methods compatible with autometallography are described. The difficulties the researcher can face by using autometallography are predicted. The ways to solve the problems are recommended. The compatibility of both methods with other immunohistochemical procedures is depicted. Two variants of enzyme metallography are considered. The mechanism of enzyme metallography is analyzed. The experimental results obtained by different research groups are summarized. The methods aimed for further signal amplification are criticized.

Keywords: autometallography, enzymatic metallography, enzyme metallography, peroxidase

Enzyme-linked immunosorbent analysis (ELISA) is a widely used analytical method thanks to simple operations, low equipment cost and at the same time its high sensitivity and selectivity. We have analyzed sensitivity of commercial ELISA kits and concluded that most kits have detection limit in the range 1–100 pM and the lowest detection limit nowadays is 100 fM.

Many proteins present in blood plasma have low concentration. One can say that only several tens of different proteins occur in the plasma at concentrations of 10^{-3}–10^{-4} M [1]. The lower the concentration, the greater is the diversity of proteins present in the plasma; there are more than 1000 proteins present at a concentration of 10^{-15} M. The existing proteomic technologies enable detection and identification of up to 10–20 % of the various protein species present in plasma. Therefore, decreasing detection limit even by 1–2 orders will significantly increase the possibilities of medical diagnostics. The application of signal amplification methods paves the way to overcome this problem.

Autometallography

Autometallography which is also called electroless deposition is an amplification method based on non-enzymatic process of germinal particle growth as a result of deposition of metal from the solution onto its surface [2].

Small silver or gold nanoparticle is being used as a germ. Developing solution containing silver or gold salt is also needed. Under these conditions metal deposits onto existing crystallization centers. Hence they grow to the necessary size. The drawbacks of this method are high background, i.e. spontaneous silver crystal formation, and photosensitivity of the chemicals being used. The method allows using different metals such as silver, gold, nickel, platinum. Silver and gold enhancement methods also called silver and gold development are the most widespread. Autometallography can be used both for growth of metal nanoparticles and for obtaining lengthy structures called nanowires [3–6].

Silver enhancement was initially used as a method of photographic development. The first biological application of this method was the detection of minute amounts of metals, including gold, in tissue sections [7]. Combination of immunogold method which is widely used in electron microscopy with silver enhancement lead to appearance of new highly sensitive type of immunoassay [8–9]. The method is of much enhanced sensitivity (up to 200-fold) as compared with peroxidase-antiperoxidase method and is compatible with common immunohistochemical procedures. It gives higher contrast and does not require using carcinogenic chemicals. The techniques used can be automatized. Metal silver which is a product of the reaction cannot diffuse, the cost of silver salts being multiply compensated thanks to decrease of antibodies due to their possible dilution [8–10]. Since autometallography gives maximal demonstration using concentrations of primary antisera well beyond the range of sensitivity of the peroxidase-antiperoxidase method, it is possible to use primary antisera raised in the same species for double immunolabeling, provided autometallography is performed first [8]. Sample antigenicity keeps safe after silver enhancement. In case of using silver enhancement in conjunction with enzymatic labeling silver enhancement should be completed before the application of the enzymatic probe. If the enzymatic probe is applied first, the substrate can act as a nucleating agent during autometallographic enhancement and give nonspecific background staining [10]. The method allows determination of antigens in whole blood without decrease of sensitivity compared to the analysis of serum [11].

Autometallography may be used for correlative microscopy: it is possible to carry out a single labeling experiment to label both at the electron microscopy and light microscopy level because identical nanoparticles can be grew to different sizes and thus to provide direct correlation between the two [2].

Autometallography may be used in double labeling experiments, so that two labels can be visualized together. For electron microscopy, two strategies are as follows. The first strategy is to silver enhance nanoparticles but make them distinguishable from some standard colloidal gold sizes. The second strategy is to silver develop the first target gold particle to larger size, then label the second target with gold and apply a second silver enhancement. Since the first gold particles will then have been developed twice, they become distinguishably larger than the second label [2].

The 1,4 nm particles are the smallest gold clusters that can be seen directly under a conventional electron microscope, allowing a spatial resolution of about 7 nm when covalently attached to antibody fragments. The visibility of the clusters can be improved by silver enhancement step for use in electron of light microscopy for histological purposes, or to detect picogram amount of antigens in immunoblots [12]. Sometimes the enlarged nanoparticle labels can be detected even by the naked eye [4].

Silver enhancement has also been applied for DNA analysis using conventional flatbed scanner as a reader. Labeling oligonucleotide targets with nanoparticle rather than fluorophore probes substantially alters the melting profiles of the targets from an array substrate. This difference permits the discriminantion of an oligonucleotide sequence from targets with single nucleotide mismatches with a selectivity that is over three times that observed for fluorophore labeled targets. When coupled with silver enhancement, the sensitivity of this scanometric array detection system exceeds that of the analogous fluorophore system by two orders of magnitude [13].

Not only electron microscopy permits to see nanoparticles directly but also atomic force microscopy does. The distance between nanoparticles just separated in the optical image can be measured with nanometer resolution by atomic force microscopy. For surface densities below 10 particles/μm^{-2}, a linear dependency of the surface density on the concentration was found [14]. However, this method has several drawbacks. It is very slow (minutes per image), it can only visualize a limited region of about 100 μm^2 at a time, and it needs equipment not available in standard biological laboratories. The application of autometallography allows sometimes doing with optical images [14].

The application of silver enhancement as a stage of sandwich immunoassay on the surface of an ultrasensitive microgravimetric biosensor based on quartz crystal microbalance result in ca. two orders of magnitude improvement in human IgG quantification [15]. Moreover, for DNA detection by analogous biosensor using gold enhancement detection limit was ~$1 \cdot 10^{-15}$ M [16].

Silver enhancement has been applied as a stage of sandwich immunoassay on a compact disc surface. The measured C-reactive protein concentration value corresponded well with that obtained from the same assays performed on glass slides [17].

Silver enhancement has been used as a part of POCKET immunoassay. This method includes antigen adsorption onto the surface of a transparent polystyrene plate which is followed by addition of antibodies conjugated to 10 nm gold colloids and subsequent silver enhancement. The resultant silver film, whose opacity is a function of the concentration of the analyte, partially blocks the transmission of light through the polystyrene plate allowing quantitative antigen determination using an optical integrated circuit as a photodetector. This circuit contains a photodiode, an amplifier, and a voltage regulator. The analytical performance of this assay approaches that of ELISA using relatively expensive bench-top equipment. The method is low-cost and portable, and therefore is appropriate for use in the field [18].

Gold enhancement has a number of advantages over silver enhancement. In addition to higher contrast in the electron microscope, greatly increased backscatter signal (for scanning electron microscopy), and resistance to osmium etching, gold enhancement gives a longer time between full development and autonucleation. Unlike silver, gold is not precipitated by chloride, and therefore gold enhancement can be conducted in the presence of physiological buffers containing saline. Compared with silver enhancement, lower backgrounds have been reported for some immunohistochemical experiments using gold enhancement. Unlike silver enhancement, which frequently requires pH 3,5, gold enhancement is being conducted at pH near 7 [10].

The silver reaction can still change even after thorough water wash. Therefore, strong light should be avoided after silver enhancement. Sodium thiosulfate (1% aqueous solution, freshly made) is a good stop reagent for both silver and gold enhancement. However, caution should be exercised with this procedure when using gold enhancement. In some experiments treatment with sodium thiosulfate has been found to reduce signal. Other solutions used for stopping autometallography include acetic acid, sodium chloride, and

photographic fixers. Successive treating a sample with several solutions is also used. When using acetic acid further decrease of background can be achieved by adding glucose to the solution.

In some procedures little or no development has been found upon autometallography. Results may be improved in these systems by changing from commercial silver enhancement reagents to freshly-prepared solutions made by yourself or by substituting formaldehyde for glutaraldehyde in postfixation [10]. If aldehyde-containing reagents have been used for fixation, these must be quenched before labeling. This may be achieved by incubating the specimens in glycine solution in phosphate buffered saline; ammonium chloride or sodium borohydride in phosphate buffered saline may be used instead of glycine [2].

Silver enhancers tend to be divided into two types. The first is often based on silver lactate, which includes a thickening agent or protective colloid, usually gum Arabic, although gelatin and polyethylene glycol (carbowax) have also been used, and is light sensitive. These may consist of three or more components, and are usually preferred for electron microscopy because they produce enhanced particles of a more uniform size and shape and allow improved preservation of ultrastructural morphology. The second type is usually not highly light sensitive, although strong illumination does have an effect, and the formulation is often based on silver acetate, although other silver salts have been used. These are simpler to use, usually consisting of two components that are mixed immediately before development and are preferred for light microscopy and blotting because development can be visually monitored. Use of a safelight is recommended for these developers, but development under a box to exclude direct light in a normally lit room is acceptable [10].

After treating samples after silver enhancement with complicated composition solutions containing complexes of ferric or cupric ions with organic ligands sample colouring occurs. The resulting colour depends on the solution used. This method allows using less amount of deposited silver for optical visualization of the label than in the case of direct silver determination using its black colour [19–20].

In the optical image, particle pairs in different states regarding their shapes can be distinguished. When the interparticle distance becomes smaller, the originally separated two particles touch each other in the optical image, resulting in an '8'-shaped outline. For even smaller distances, this outline shifts to a more elliptical shape [14]. The smallest distance allowing distinguishing the particles with the help of the optical system was about 400 nm.

Gold nanoparticles conjugated with oligonucleotides can bind to the surface of silver deposited on analogous conjugates by silver enhancement and serve as crystallization centers at the second silver enhancement stage. Unbound conjugates can be washed out with water to prevent additional background increase. Sample drying with air does not influence specificity of the consequent silver enhancement. The process may be repeated as often as desired to further enhance the amount of deposited silver and the darkness of the spot. With this amplification method, one can readily observe a dark silver spot for an assay using 25 μM target concentration. With two cycles of the new nanoparticle-silver sandwich procedure, 1 fM target solutions can be recognized, and with three cycles, 0,1 fM solutions give positive though weak spots. The method is compatible with using commercial silver enhancement kits [21].

The control that covalent labeling provides over probe architecture and configuration has led to the development of combined fluorescent and gold probes. Selective coupling of the gold cluster to a unique site in an antibody fragment, such as a hinge thiol, allows the attachment of a fluorescent label elsewhere on the antibody via a second cross-linking reaction to yield a probe with both fluorescent and gold labels. These probes can be used for correlative fluorescence and electron microscopy or for checking labeling by fluorescence microscopy before undertaking electron microscopy processing [2].

An important consideration when designing such probes is fluorescence resonance energy transfer. Calculations with the gold cluster which is 1,4 nm in diameter yield a Förster distance of approximately 6 nm, allowing usable fluorescence when both labels to be linked to a single antibody. However, Förster distances increase significantly for larger colloidal gold particles. Conjugation of the fluorescent label to a second antibody which binds to the gold-conjugated one increases the gold-fluorophore separation to 10–20 nm; although quenching is still present, sufficient fluorescence is restored for observation by CCD camera [2].

Enzyme metallography

Enzyme metallography (enzymatic metallography, EnzMet™) deals with precipitation of metals such as silver, gold, iron, mercury, nickel, copper, platinum, palladium, cobalt and iridium from solution as a result of enzymatic reduction [2, 22–24]. Enzyme metallography is a promising method for medical diagnostics particularly because of its possibility to determine single antigenic determinants. It is a widespread method for determination

of oncomarkers [22]. Enzyme metallography results in formation of dark dense metal precipitates. There are three main types of enzyme metallography.

The precipitation of metal nanoparticles by means of polymeric matrices

This type of enzyme metallography deals with metal nanoparticles bound to the molecules of substrate which enzymatically forms precipitate. Nanoparticles are also being incorporated into this precipitate. Gold nanoparticles which are 1–3 nm in diameter and functionalized by different ligands (4-hydroxythiophenol, 3,3′-diaminobenzidine, glutathione) in presence of 0,03–0,06 % hydrogen peroxide form precipitate as s result of the reaction catalyzed by horseradish peroxidase (HRP). For precipitation of nanoparticles stabilized by thioglucose extra 0.25 % hydroquinone is needed. The method is patented (Hainfeld et al., USA) [25–27].

Enzymatic generation of metal ions reducing agent

This type of enzyme metallography deals with enzymatic generation of active reducing agent from an inactive precursor. The product being formed near enzyme molecules reduces silver or gold ions in solution. The method is patented (Bieniarz et al., USA) [28–30].

Enzymatic generation of reducing agent has been applied for DNA determination. This approach deals with hybridization of target DNA with one immobilized on the electrode surface and subsequent hybridization with biotinylated DNA. After hybridization avidin conjugated alkaline phosphatase was added, which binds to the exposed biotin group on the detection probes and converts *p*-aminophenylphosphate to *p*-amonophenol, a reducing agent that reduces silver ions. The extent of hybridization was measured electrochemically by the magnitude of anodic current because the precipitation of silver on the electrode surface increases its surface area. The precipitation of silver on electrode surface was proved by quartz crystal microbalance. Compared with the results obtained by in situ surface plasmon resonance, this method gave 3-fold improvement in the hybridization detection [31].

Enzymatic reduction of metal

This type of enzymatic metallography deals with change of reaction specificity of horseradish peroxidase with the result that the enzyme catalyzes the reduction of different metals from a solution. The method is patented (Hainfeld et al., USA) [25-27].

It is known that horseradish peroxidase exhibits NADH oxidase [32-33], NADPH oxidase and thioloxidase activity [34] besides its normal peroxidase activity. In thiol oxidation oxygen is the oxidizing agent [34]. As for NADH and NADPH oxidation, oxygen and hydrogen peroxide both are oxidizing agents [35].

When oxygen is introduced continuously into a solution of NADH and horseradish peroxidase the oscillatory consumption of oxygen can be observed. Under suitable experimental conditions the aerobic oxidation of NADH catalyzed by horseradish peroxidase occurs in four characteristic phases: initial burst, induction phase, steady state and termination. A trace amount of hydrogen peroxide present in the NADH solution is enough to bring about initial burst [32–33].

In the presence of ethylenediaminetetraacetic acid (EDTA) the enzyme exhibits also reductase activity [36]. Oxidation and reduction reactions catalyzed by horseradish peroxidase have different pH optima. They are pH 3.5 for peroxidase activity and pH 6 for reductase activity [37].

Spectroscopic studies have shown that horseradish peroxidase substrates can be divided into two classes on the basis of the difference spectra observed when they bind to the enzyme [38]. Normal peroxidase substrates such as phenol, p-cresol, hydroquinone and aniline are in the first class. Guaiacol and resorcinol are in the second class. The physical basis for this spectroscopic classification is obscure [38].

It was found that lignin peroxidase also displays resembling activity. This enzyme also catalyzes the reduction of different electron acceptors such as cytochrome c, nitro blue tetrazolium, ferric ion and molecular oxygen in a reaction mixture containing iodide, hydrogen peroxide and EDTA [39]. The activity observed using iodide as a mediator was comparable to that obtained using other free radical mediators, such as veratryl alcohol and a variety of methoxybenzenes. Other peroxidases, such as horseradish peroxidase, lactoperoxidase, myeloperoxidase and gastric peroxidase, were also found to catalyze the same reactions. Cloroperoxidase catalyzes the same reaction at a relatively higher rate than horseradish peroxidase. Lactoperoxidase also possesses the same property but requires higher EDTA concentration. Despite of its more complex protein and heme structure its reductase activity resembles that of horseradish peroxidase, however, the mechanisms are somewhat different [40]. Peroxidases use not only iodide but bromide too, myeloperoxidase using also chloride. The results for horseradish peroxidase were analogous [39, 41]. However, the behaviour of lignin peroxidase and horseradish peroxidase under identical conditions is highly different [42].

Hemoglobin and cytochrome c also catalyze the reaction but at a much slower rate, possibly due to their pseudoperoxidase activity. Albumin does not possess the property, indicating that the intact heme part bound to the protein is necessary for the reaction.

EDTA in concentration 4 mM blocks horseradish peroxidase catalyzed I^- oxidation to I_3^- that is shown by lack of increase of solution absorption at 353 nm. At the same time 2 mM EDTA while blocking direct reaction causes reverse reaction, i.e. I_3^- reduction that is shown in turn by decrease of solution absorption at 353 nm. Both enzymatically obtained (before EDTA addition) and chemically obtained I_3^- as well as iodine or I^+ can be reduced. Turnover number determination showed that I^+ or I_2 reduction is significantly more efficient than I_3^- reduction. Thus positively charged or non-polar oxidized substrates appear to be better than negatively charged I_3^-. Iodide present in this complex appears to modulate reductase activity of horseradish peroxidase [37]. In addition, hydrogen peroxide is being oxidized to oxygen (pseudocatalatic reaction). Reversibility of the effect caused by EDTA shows that enzyme inactivation does not proceed. Both direct and reverse reactions are being inhibited by azide which is an irreversible peroxidase inhibitor [36].

Horseradish peroxidase contains two Ca^{2+} ions per enzyme molecule. Only one tightly bound Ca^{2+} could be removed by very drastic treatment such as incubation with EDTA or [et hylenebis(oxyethylenenitrilo)]tetraacetic acid in the presence of guanidinium chloride, as this Ca^{2+} is essential for maintaining the protein structure in the haem cavity for enzyme activity. The observed inhibition is not due to removal of this intramolecular Ca^{2+} by EDTA as the drastic conditions required to obtain Ca^{2+}-free enzyme were not used. Insensitivity to [ethylenebis(ox yethylenenitrilo)]tetraacetic acid also supports this view. Moreover, the effect of EDTA is not reversed by addition of Ca^{2+}, suggesting that this Ca^{2+} is not chelated by EDTA [42].

In order to get an idea of which part of the EDTA structure is essential for the induction of catalytic conversion of iodine to iodide, the effects of various EDTA analogues were investigated [42–43]. N, N, N`, N`-tetramethylethylenediamine shows very close resemblance to the structure of EDTA and was 80% as active as EDTA. It appears that the CH_2COOH groups of EDTA are not necessary as the same effect could be obtained when they are replaced by the methyl groups. However, a possible role of the carboxy groups of EDTA in the electrostatic interaction with some positively charged groups at the haem distal pocket cannot be excluded [42].

EDTA inhibition is reversed by presence of equimolar concentrations of Zn^{2+}, Ga^{2+}, and Cd^{2+} which are chelated via the lone pairs of electrons on the nitrogens, and not by Ca^{2+} or Mg^{2+}, which are chelated through the carboxy groups [36, 42–43]. This indicates that although the COOH groups of EDTA are not necessary, the two nitrogens appear to be essential. A fixed chain length between the two nitrogens also appears to be essential. An increase in the chain length by the glycol group reduces the effect by 90%. Ethylenediamine, a secondary amine having a chain length similar to EDTA, is absolutely ineffective, suggesting that the structure in the tertiary amines like EDTA or N, N, N`, N`-tetramethylethylenediamine is suitable for interaction with the enzyme. Although triethylamine is a tertiary amine, the absence of another nitrogen atom in its structure makes it ineffective. Ethylenediaminediacetic acid, which resembles EDTA but has two carboxyl groups less, is 62% as effective as EDTA [42].

Based on absorption and circular dichroism spectra of compound I in the presence of EDTA as well as iodide displacement from its complex with peroxidase by EDTA the authors of [43] concluded that the interaction of EDTA at the iodide-binding site leads to a change in heme conformation associated with the modification of the catalytic activity of the enzyme.

It was shown that the inhibition constant value for EDTA inhibition of iodide oxidation by the catalytically active lactoperoxidase at its optimum pH is smaller than the binding constant value for the binding of EDTA to the native enzyme at the same pH. This indicates that the catalytically active lactoperoxidase (compound I and II) binds EDTA at higher affinity than the native enzyme presumably due to higher oxidation-reduction potential between the active enzyme and EDTA, which attracts EDTA for interacting at the active site. Similar explanation has been given in case of iodide oxidation and iodide binding by horseradish peroxidase [40].

Cyanide binds irreversibly to haem iron. Based on spectral data the authors of [37] concluded that EDTA binds to this complex. The binding constant value for the binding of EDTA to the HRP-CN complex is close to that obtained for the binding of EDTA to the native enzyme. Thus EDTA appears to bind away from the haem iron centre.

The binding of EDTA to horseradish peroxidase is weaker than that of cyanide. It was shown that EDTA binding significantly decreases in the presence of iodide, nitrite or thiocyanate. According to the authors of [37] this effect can be explained by their competition for the same binding site. High iodide concentrations reverse the EDTA-induced block of iodine oxidation and at the same time inhibit the iodine reductase activity. Thiocyanate could

not be used as it reduces iodine non-enzymatically. This is not a non-specific effect of high salt concentration as nitrate has no such effect under identical conditions.

A plot of log K_D for the HRP-EDTA complex against pH produced a sigmoidal curve. The binding constant value in the near-saturation field indicates that an ionizable group on the enzyme with a pK_a of 5,8 is responsible for controlling the binding of EDTA to the enzyme and this is presumably the distal histidine [37]. In the case of lactoperoxidase the possible role of the distal histidine in binding of iodide and thiocyanate and subsequent electron transfer to the heme ferryl group has been suggested [40].

To determine different horseradish peroxidase substrate binding sites and oxidation mechanism details δ-meso-ethylheme-reconstituted peroxidase was used [38]. It was shown that two-electron oxidation of thioanisoles to the corresponding sulfoxides catalyzed by horseradish peroxidase proceeds with considerable enantioselectivity. The electronic properties of the substrate do not significantly contribute to the reaction enantioselectivity. Racemic sulphoxides can be formed up to 4% of the enzymatic rate by direct reaction with hydrogen peroxide. The modified peroxidase is inactive toward guaiacol oxidation despite the fact that it appears to react with hydrogen peroxide. A δ-meso-ethyl group also virtually blocks iodide oxidation but actually increases sulfogenase activity. However the reconstituted enzyme is far less stereoselective.

Reconstitution of the enzyme with hemin did not result in loss of stereoselectivity, so that the loss observed with δ-meso-ethylheme is unlikely to be an artifact of the reconstitution process.

Heat-denatured horseradish peroxidase retained 3% of its ability to stimulate guaiacol oxidation and 15% of its ability to catalyze sulfoxygenation, but the resulting sulfoxide product is racemic. Control incubations with no enzyme did not support guaiacol peroxidation but did support slow oxidation of thioanisole to racemic sulfoxide. The oxygen incorporated into the sulfoxide in the horseradish peroxidase-catalyzed oxidation of thioanisoles derives primarily from the peroxide. The values obtained for the incorporation of $H_2{}^{18}O_2$ oxygen into the sulfoxide when the reaction was catalyzed by native and δ-meso-ethylheme-reconstituted horseradish peroxidase were exactly the same. This provides clear evidence that the mechanisms of the sulfoxidation reactions catalyzed by native and modified peroxidase are also the same [38].

All heme proteins with an accessible iron atom react with phenyldiazene to form a spectroscopically detectable phenyl-iron complex. The fact that horseradish peroxidase does not form such a complex is one of the pieces of evidence that argues that its iron atom is not accessible to substrates. Spectroscopic examination of the reaction of δ-meso-ethylheme-reconstituted horseradish peroxidase with a large excess of phenyldiazene shows that the modified enzyme also does not form a phenyl-iron complex. The δ-meso substituent therefore does not perturb the active site structure sufficiently to make the heme iron available to phenyldiazene.

The reaction of phenylhydrazine or phenyldiazene with horseradish peroxidase results in covalent binding of two phenyl residues to the protein and loss of guaiacol oxidizing activity. The loss of this activities correlates with protein modification rather than with the minor degree of δ-meso-phenyl heme substitution that also occurs. Phenylhydrazine-modified horseradish peroxidase is also inactive toward the oxidation of iodide but is considerably more active, although less enantioselective, toward thioanisole sulfoxidation than native horseradish peroxidase. Phenylhydrazine treatment also does not inactivate the porphobilinogen oxygenase activity of horseradish peroxidase.

Absorption spectra show that guaiacol binds to modified peroxidase even if it is not being oxidized. δ-meso-Methylheme-reconstituted horseradish peroxidase retains guaiacol oxidizing activity but is more sensitive to peroxide-dependent inactivation. In contrast to guaiacol oxidation, which is slightly stimulated by a δ-meso-methyl substituent, iodide oxidation and thioanisole sulfoxidation are partially inhibited. The lower activity with the δ-meso-methylheme may be due to the lower stability of the enzyme reconstituted with this prosthetic group and the relatively long incubation times required for sulfoxidation. This explanation is supported by the fact that the chromophore of the δ-meso-methyl substituted enzyme is lost more rapidly than that of the δ-meso-ethyl substituted enzyme. The sulfoxidation enantioselectivity, however, is decreased by the δ-meso-methyl as well as δ-meso-ethyl groups [38].

Kinetical data clearly indicate that iodide does not inhibit guaiacol oxidation by competing for a common binding site. Inhibition of guaiacol and iodide oxidation by thioanisole is also unlikely to result from competition. These conclusions were confirmed by absorption spectra. EDTA inhibits oxidation of both iodine and guaiacol competitively [42].

I^+ binding in the HRP-CN complex indicates that its site is away from the heme iron center. I^+ binding remains unaltered by guaiacol or vice versa, suggesting that I^+ binds away from the aromatic donor binding site. A plot of

log K_D of I^+ binding against various pHs shows the involvement of an ionizable group on the enzyme having pK_a = 4.8, contributed by an acidic group, deprotonation of which favors I^+ binding. As I^+ reduction occurs at a saturating concentration of EDTA, I^+ binding at the EDTA site could be excluded [44].

However, in the case of lactoperoxidase EDTA binds close to the iodide binding site but it significantly alters the aromatic donor binding also. This indicates that either iodide and aromatic binding sites are very close to each other so that binding to iodide site disturbs the aromatic donor binding or EDTA binds in between the iodide and aromatic donor sites. In both cases, however, these sits should be very close at the heme edge [40]. NMR experiments suggest that iodide and thiocyanate bind to a common site roughly equidistant from the 1 and 8 heme methyl groups and approximately 6-10 Å from the iron atom [38].

ESR studies showed the presence of EDTA radical in the reaction mixture. This radical is a product of EDTA oxidation by iodide radical [41] or directly by the peroxidase only [42]. This radical can reduce iodine. The authors of [41] insist that EDTA radical can reduce cytochrome c to ferrocytochrome c and molecular oxygen to O_2^-, while these results were not confirmed in the article [42]. The inhibition of reduction activity at high concentrations of iodide might be due to the combination of iodide radicals to form iodine, which forms a stable triiodide complex in the presence of excess iodide [41]. However, the evidence for the formation of the iodine radical is lacking [42].

It was found that in the reaction mixture containing radiolabeled [acetic-1-^{14}C]EDTA, iodide and hydrogen peroxide EDTA decarboxylation by hypoiodite proceeds. Some EDTA was also decarboxylated in the absence of the enzyme as the reaction mixture contained hydrogen peroxide and iodide, which can generate hypoiodite. It was shown that chemically synthesized hypoiodite also causes EDTA decarboxylation. Both iodide and hydrogen peroxide can reduce hypoiodite [43]. It is supposed that hypoiodite is a product of iodide oxidation by compound I [39]. In the case of horseradish peroxidase EDTA decarboxylation is not evident, and EDTA concentration remains the same throughout the reactions [44].

The participation of superoxide as an intermediate in the mechanism is supposed [45]. Potassium superoxide or a biochemical superoxide generating system can reduce iodine to iodide. Superoxide can be a product of hydrogen peroxide oxidation by compound I in the presence of EDTA or that of compound III dissociation. Superoxide dependent iodine reduction can be direct or mediated by EDTA-Fe complexes.

EDTA-Fe(III) complex mediates reactions between active oxygen species and can be reduced by superoxide. EDTA-Fe(II) complex can reduce iodine and form triiodide. It is known that EDTA preparations contain trace amounts of iron [45].

The alternative iodine reduction mechanism without superoxide radical participation has been suggested [44]. In the presence of I^+ a ternary complex of compound I-I^+-EDTA is formed, which generates compound II-I·complex and both nitrogen-centered dication radical (N^+-N^+) through intermolecular electron transfer from EDTA nitrogens. Compound II-I·complex is further reduced similarly by another molecule of EDTA to form ferric enzyme, iodide ion and EDTA dication radical. The dication radical may be released from the active site and, being more reactive, oxidizes hydrogen peroxide to oxygen at a faster rate to regenerate EDTA. The existence of dication radical is suggested from the similarity of its ESR signal with that of single nitrogen-centered monocation radical [44].

The authors of [46] have obtained horseradish peroxidase redox intermediates in crystal state. They have obtained their X-ray images and presented the mechanism of bound oxygen reduction by X-ray irradiation catalyzed by peroxidase.

The application of enzyme metallography

The main examples of possibilities of enzyme metallography were connected with oncomarkers and their genes determination. In spite of that enzyme metallography can be applied in many other fields of biochemical analysis.

Enzyme metallography has significant advantages when compared to conventional peroxidase-diaminobenzidine system forming colored polymeric precipitates. Unlike this system, enzyme metallography leads to extremely low diffusion of dense reaction products, which can be easily determined. Therefore, it gives possibility to distinguish cell compartments with high resolution. High density of silver precipitated allows much more distinct cell structure visualization and permits evaluation of core immunophenotype at a relatively low magnification, allowing more tissue to be screened in an efficient manner [47]. Besides, it is the most precise quantitative method having simple experimental protocols and because of it being suitable for automatization. Enzyme metallography procedures do not influence results obtained using *fast red K* dye [22]. The detection system is applicable to either bright field in situ hybridization or immunohistochemistry [47]. The only disadvantage of the method is impossibility to visualize cell structures using several colours.

The combination of determination of gene and protein encoded by this gene by enzyme metallography (EnzMet GenePro) was applied to determination of oncoprotein HER2 gene amplification [48]. This approach had a sensitivity of 82,9%, a specificity of 100%, and an accuracy of 92,6% according to FDA guidelines. The actual scoring of the slides was done without the use of oil immersion microscopy. Correlation between immunohistochemical component of the assay and the HER2 gene amplification status of the assay had agreement in 98% of the cases. Moreover, the single gene copies are distinctly identified as black signals. This fact allows us to conclude that enzyme metallography permits to visualize single molecules.

The method has also been applied to in situ DNA hybridization. The paper [49] deals with biotinylated DNA determination by hybridization with DNA immobilized between electrodes followed by streptavidin-peroxidase addition and enzyme metallography. The method permits to detect single mismatches. Unlike silver enhancement, it has extremely low background [24, 49–50] which can be decreased further by preliminary washing samples with sodium periodate, 1% sodium thiosulfate or Lugol's iodine.

There is a possibility of further signal amplification by autometallography on silver or gold germs obtained by enzyme metallography [49]. However, this approach leads to increased background. Therefore, additional enzyme metallography stage after washing appears to be more promising. The drying stage after washing must be excluded to prevent enzyme inactivation.

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