Anisotropic Orientation of Horseradish Peroxidase by Reconstitution on a Thiol-Modified Gold Electrode

Heiko Zimmermann,*[a] Annika Lindgren,*[b] Wolfgang Schuhmann,[a] and Lo Gorton[b]

Abstract: Horseradish peroxidase (HRP) was reconstituted on the surface of a gold electrode that was modified first with a hemin–carbon-chain–thiol derivative followed by addition of the apo protein to the contacting solution. To facilitate the reconstitution of the holo enzyme, the hemin needs to be immobilised on a carbon-chain spacer arm. To achieve this, an immobilisation protocol was developed that is based on the initial formation of a mixed self-assembled monolayer on the gold surface consisting of 3-carboxypropyl disulphide and an activated disulphide (3,3′-dithiodipropionic acid di-(*N*-succinimidyl ester)) followed by binding of a diaminoalkane to the activated disulphide. The hemin was then coupled to the second amino group of the diaminoalkane by means of a carbodiimide coupling reagent. Finally, the enzyme was reconstituted on the hemin-modified surface by immersion of the electrode in a solution containing apo-HRP. The advantage of this method is that the length of the spacer arm can be changed easily, because diaminoalkanes of different chain lengths are available. The electrochemistry of the hemin and the reconstituted HRP electrodes was studied by means of cyclic voltammetry and differential-pulse voltammetry. The catalytic ability for reduction of hydrogen peroxide was investigated for both direct and mediated electrochemistry with a soluble electron donor (*ortho*-phenylenediamine).

Keywords: heme proteins · horseradish peroxidase · monolayers · protein immobilisation · redox chemistry

Introduction

An increasing interest in the development of reagentless biosensors has focused research on redox enzymes that are capable of direct electron exchange with suitable electrode surfaces without free-diffusing redox mediators. The prerequisites for a direct electron transfer (ET) can be derived from the Marcus theory,[1, 2] which demonstrates the key importance of the ET distance, besides the potential difference and the reorganisation energy of the redox centres involved. As a consequence, for an optimally designed electrode configuration one has to ensure that the ET distance between an immobilised redox protein and an electrode surface is as short as possible. Hence, only those enzyme molecules that are immobilised in the first monolayer on an electrode surface are able to undergo direct ET.

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One of the most intensively studied and best characterised group of enzymes exhibiting direct ET characteristics are the peroxidases, such as cytochrome *c* peroxidase,[3] horseradish peroxidase (HRP),[4] fungal peroxidase,[5, 6] lactoperoxidase,[7] microperoxidase[8, 9] and chloroperoxidase[10] immobilised mainly on carbonaceous materials and noble metals. The characteristics of various biosensor designs that utilise these enzymes have been summarised recently.[11, 12] A common aspect of these investigations was that the enzymes were directly immobilised on the electrode surface, usually simply by adsorption. For example, random adsorption of native HRP on graphite electrodes has the result that about 40–50% of the remaining active enzyme molecules are in direct ET contact with the electrode.[13, 14] Additionally, redox proteins had been pre-orientated by means of promoter-modified electrode surfaces favouring the right orientation of the protein and thus facilitating the direct exchange of electrons following an adsorption–reaction–desorption mechanism.[15, 16]

The ET distance depends on the overall distance between the redox site within the protein and the electrode surface, the depth of the active site inside the protein and the orientation of the protein on the electrode surface. The last two factors are also correlated with the size of the protein itself. To define the distance between the electrode surface and the immobilisation site, self-assembled monolayers (SAM) spontaneously

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formed from solutions of thiol derivatives on clean gold surfaces[17–19] can be used. They may also provide suitable functional groups for covalent attachment of redox proteins.

Orientation of small heme-containing redox proteins like microperoxidase MP-11[20–22] on cystamine-modified gold electrodes revealed close to reversible electrochemistry for $Fe^{3+/2+}$ and high electrocatalytic activity for the reduction of H_2O_2. It has also been shown that hemin can be successfully tethered to a cystamine-modified gold electrode.[23]

One would not expect similar behaviour for an analogously prepared HRP electrode, since the size of the protein together with an isotropic orientation prior to covalent fixation would severely decrease the number of molecules bound in a favourable orientation required for direct ET reactions. Thus, especially for large proteins, the overall ET distance can only be decreased by attempting an anisotropic orientation of the protein prior to covalent immobilisation with the active site exposed towards the electrode surface. Examples of this strategy are the reconstitution of holo oxidases with thiol-derivatised FAD,[24–26] of PQQ-dependent dehydrogenases with PQQ-modified thiol monolayers[27] and of myoglobin by means of monolayers of thiol-derivatised hemin.[28]

In this paper, we report the design and evaluation of an electrode architecture for orientated immobilisation of HRP on a SAM; this procedure circumvents tedious syntheses of cofactor-derivatised thiol compounds that possess the appropriate spacer length, while the flexibility to adapt the distances to the specific needs of envisaged direct ET processes is maintained.

Abstract in German: *Durch Zugabe des Apo-Enzyms wurde Meerrettichperoxidase (HRP) auf einer Hämin-funktionalisierten Thiolmonoschicht orientiert rekonstituiert, um die Elektronentransferdistanz zwischen dem Cofaktor des Redoxproteins und der Goldelektrodenoberfläche zu minimieren. Die Architektur der Hämin-funktionalisierten Tiolmonoschicht wurde so konzipiert, dass die Distanz zwischen Hämin und Alkylthiolmonoschicht über Spacerketten variabler Länge eingestellt werden konnte, während gleichzeitig eine laterale Verdünnung der Hämin-Ankergruppen erreicht wurde. Hierzu wurde im ersten Schritt eine gemischte Monolage aus einer Lösung von 3-Carboxypropiondisulfid und einem Aktivesterderivatisierten Disulfid (3,3′-Dithiodipropionsäuredi(N-hydroxysuccinimidyl)ester) in DMSO auf der Goldelektrode gebildet, wobei das Verhältnis Aktivester- zu Carboxylatfunktion frei gewählt werden kann. An die Aktivestergruppen wurden im zweiten Schritt 1,ω-Diaminoalkane gekoppelt und anschließend Hämin nach Carbodiimid-Aktivierung an die noch freie Aminogruppe gebunden. Im letzten Schritt wurde das Holo-Enzym auf der Hämin-modifizierten Oberfläche in einer Lösung von Apo-Peroxidase rekonstituiert. Der so erhaltene modulare Aufbau ist prinzipiell auf weitere Cofaktoren und ihre zugehörigen Redoxproteine übertragbar. Die Elektrochemie von Hämin und der rekonstituierten HRP wurde mittels cyclischer Voltammetrie (CV) und Differenz-Pulsvoltammetrie (DPV) untersucht. Die katalytische Aktivität der erhaltenen Elektroden hinsichtlich der Reduktion von Wasserstoffperoxid wurde amperometrisch in Abwesenheit (direkter Elektronentransfer) und Anwesenheit eines gelösten Elektronendonors (o-Phenylendiamin) überprüft und so die biologisch aktive Rekonstitution von Holo-HRP auf der Elektrode nachgewiesen.*

Results and Discussion

Electrode architecture for the orientated reconstitution of HRP: Since hemin located within the active site of HRP is not covalently bound to the protein backbone, it can be easily extracted to obtain the respective apo protein.[29] Moreover, reconstitution of the holo enzyme occurs easily by mixing the apo enzyme with hemin. Based on these considerations and the prerequisites for obtaining direct ET between an immobilised redox protein and the electrode surface, covalent binding of hemin at the surface of a SAM followed by reconstitution of holo-HRP with the surface-bound hemin groups was envisaged. However, according to considerations of Guo et al.[28] concerning the reconstitution of myoglobin with monolayers of hemin-modified thiol derivatives, an optimal electrode architecture has to prevent nonspecific adsorption of the protein and steric hindrance. This can be achieved by optimising the intermolecular spacing between heme moieties on the surface to distances that are at least equal to the size of the protein.[30] Additionally, in order to optimise the overall ET distance taking into account the depth of the active site of the protein, it is essential to be able to vary the spacer length between the thiol group and the heme while, at the same time, avoiding difficult syntheses of the necessary variety of hemin-modified thiol derivatives.

As a working hypothesis, a reaction sequence is proposed (Figure 1) that starts with the formation of a mixed thiol monolayer from two different thiol derivatives, one of which has an activated functional group in the ω-position. Secondly, a bifunctional spacer of variable length is bound to the activated headgroups, followed by covalent attachment of hemin. In the third step, apo-HRP is reconstituted by use of the hemin moieties attached to the surface.

For each step we have to ensure that the envisaged electrode architecture can be controlled so that nonspecific adsorption, nonproductive cross-linking of the bifunctional spacer and so forth can be prevented. In order to prove that hemin does not adsorb nonspecifically on amino-functionalised thiol monolayers, gold electrodes have been modified with a cystamine monolayer. Adsorption versus covalent binding was then assessed by comparison of electrodes that were prepared by incubation of cystamine-modified electrodes in hemin solution in the presence or absence of carbodiimide. After thorough rinsing of the electrodes in a 100 mM $NaClO_4$ solution in DMSO to desorb possibly adsorbed hemin molecules, cyclic voltammetry in 20 mM phosphate buffer (pH 7.0) was performed (Figure 2). Electrochemistry of surface-bound hemin can only be observed in the case of the carbodiimide activation (solid line in Figure 2). The monolayer-bound hemin exhibits well-behaved electrochemistry with a formal potential ($E^{\circ\prime}$) of -275 mV relative

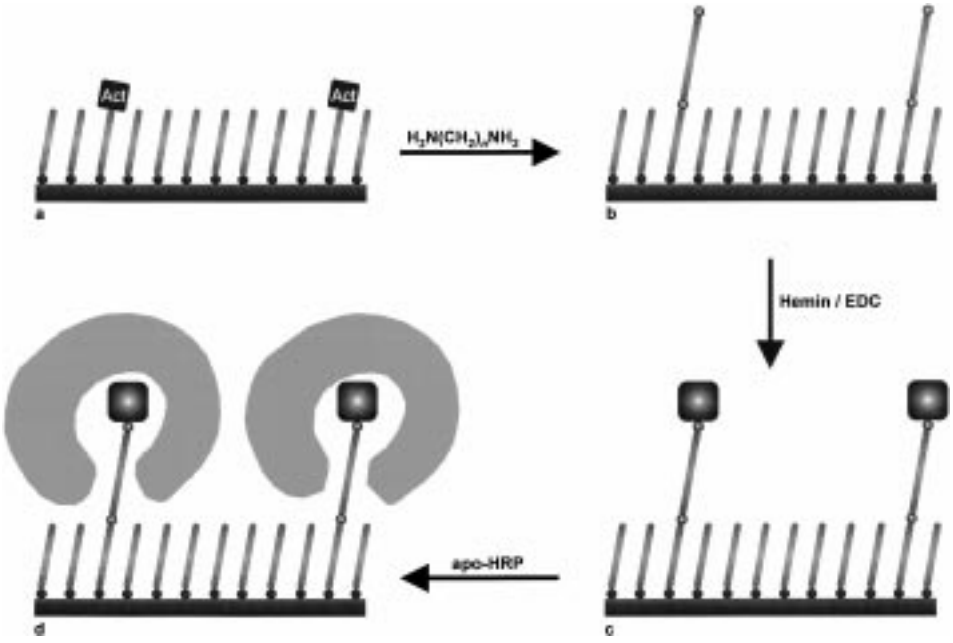

Figure 1. Schematic representation of the proposed electrode architecture for the immobilisation of hemin on a diluted spacer and the reconstitution of HRP on a monolayer-modified gold electrode. a) Formation of a mixed thiol monolayer to obtain a suitable dilution of active headgroups. b) Covalent fixation of a bi-functional spacer to the active headgroups at the monolayer. c) Carbodiimide-activation of carboxylic residues at hemin and covalent binding to functional groups at the spacer arms. d) Reconstitution of holo-HRP.

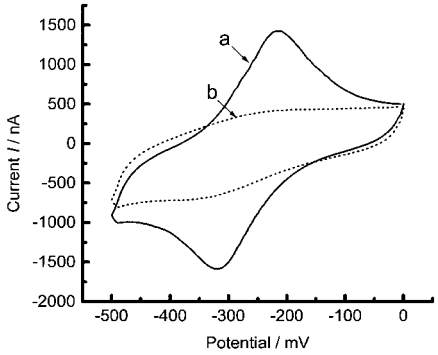

Figure 2. a) Cyclic voltammogram of hemin carbodiimide (EDC) coupled to a cystamine-modified gold electrode. b) Cyclic voltammogram of a similar electrode that was prepared without any EDC coupling reagent in the incubation solution. (20 mM phosphate buffer pH 7.0; scan rate: 100 mV s^{-1}.)

to Ag/AgCl, which was taken as the mean value of the anodic and cathodic peak potentials. These results are in accordance with those reported previously.[23] In this case, however, the hemin molecules are located too close to each other and too close to the electrode surface, thus preventing the reconstitution of a large holo protein.

Variation of the spacer length to allow penetration of surface-bound hemin groups into the active site of the apo enzyme: According to the recently elucidated structure of recombinant HRP C,[31] the distance between the carboxylic acid groups of the heme in the enzyme's active site and the surface of the protein can be estimated to be approximately 10 Å. Since the structure was derived from crystals of the deglycosylated enzyme, the depth of the active site may be even larger in native HRP. As a consequence, to accomplish the reconstitution of the active enzyme on a hemin-modified electrode surface, the distance between the electrode surface and the hemin groups should be substantially larger than that obtained for the cystamine-modified electrode. However, to facilitate direct ET the distance should be as short as possible; this means that an optimisation procedure that compromises these conflicting prerequisites is required. For an easy variation of the spacer length a three-step modification procedure has been developed. In the first step, a monolayer consisting of an activated disulphide (3,3′-dithiodipropionic acid di-(*N*-succinimidyl ester); Lomant's reagent) mixed with 3-carboxypropyl disulphide (1:99) was self-assembled on the electrode surface. Addition of a high excess of 1,ω-diaminoalkanes to the monolayer, which contained the activated head groups, yielded a monolayer terminated with amino groups (mixed with shorter chains terminated by carboxylic acid headgroups). The overall spacer length in this monolayer depends on the length of the diaminoalkanes used. We assumed that the high excess of diaminoalkane would prevent the formation of surface loops between adjacent carboxylate headgroups. The carboxylic residues of hemin were then activated with carbodiimide and covalently linked to the terminal amino groups of the monolayer by formation of amide bonds.

The electrochemical characteristics of hemin bound to diaminoalkanes of different lengths (1,4-diaminobutane, 1,6-diaminohexane, 1,12-diaminododecane) was studied using differential pulse voltammetry (Figure 3). The corresponding data are summarised in Table 1.

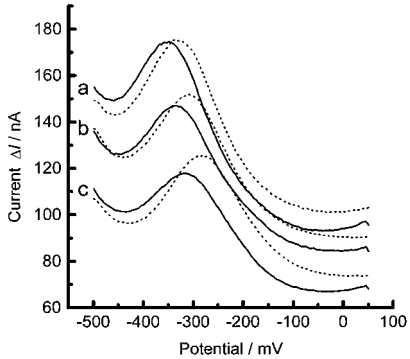

Figure 3. Differential pulse voltammograms of hemin-modified electrodes with different chain lengths of the 1,ω-diaminoalkane. a) 1,4-diaminobutane. b) 1,6-diaminohexane. c) 1,12-diaminododecane. The solid line shows the reduction peak and dotted line the oxidation peak. (Scan rate 10 mV s^{-1}, pulse height 50 mV, 1% activated disulphide.)

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Table 1. Peak separation (ΔE_p) and formal potential ($E^{\circ\prime}$) evaluated from differential pulse voltammetry experiments with different chain lengths of the diaminoalkane (4, 6 and 12 carbon atoms). The standard deviation obtained for three electrodes prepared on two different days are given in parentheses. For experimental conditions, see Figure 4.

Chain length	$E^{\circ\prime}$ [mV]	ΔE_p [mV]
4	– 327.3 (5.4)	12.7 (5.9)
6	– 310.2 (5.8)	16.6 (1.0)
12	– 291.0 (2.2)	30.7 (7.9)

The peak area decreases with increasing spacer length between hemin and the electrode. Concomitantly, with longer chains $E^{\circ\prime}$ is shifted to more positive values and the peak separation, ΔE_p, increases (Table 1). This is attributed to more rigid binding of the hemin moieties for shorter chains, leading to a voltammogram indicative of a surface-bound species, while long spacer chains allow for a longer distance, on average, of the hemin groups from the electrode surface (Figure 4). Hence, a lower electron transfer rate is obtained that leads to an increase in the oxidation peak potential and simultaneous decrease in the reduction peak potential (Figure 3 and Table 1). Taking into account possible whip-like swinging of the spacer-bound hemin group, especially for longer spacers, the redox reactions observed for surface-bound hemin with different spacer lengths do not correlate directly with defined ET distances.

Mixed monolayers as a means for optimising the distance between surface-bound hemin groups: Another important aspect is that an appropriate distance is required between tethered hemin groups to accommodate the reconstituted enzyme. The distance between the immobilised hemin molecules was adapted by means of a mixed-monolayer approach. The monolayer formed by the activated disulphide (3,3′-dithiodipropionic acid di-(*N*-succinimidyl ester)) was diluted with 3-carboxypropyl disulphide. The dilution of the activated headgroups was varied by using different ratios of the activated disulphide to 3-carboxypropyl disulphide (100, 50, 10, 5 and 0 %). After monolayer formation, the electrodes were incubated with an excess of 1,12-diaminododecane in a first step and with hemin in the presence of carbodiimide in a second step. In Figure 5 (top) the cyclic voltammograms of five hemin-modified electrodes are compared with respect to ratios of the activated and nonactivated headgroups in the initially formed thiol monolayer. The surface coverage of hemin (Γ) was calculated by integration of the peak areas obtained in the cyclic voltammograms and plotted against the ratio of activated disulphide to 3-carboxypropyl disulphide (Figure 5, bottom). A coverage of 66 pmol cm^{-2} was calculated for nondiluted activated disulphide. This value is in good agreement with the theoretical monolayer coverage for hemin, which can be derived to be 70 pmol cm^{-2} assuming an area requirement of 2.38 nm^2 per hemin molecule.[32] The linear trend in the correlation between surface coverage and dilution of activated headgroups demonstrates that the self-assembling process is under control. However, since the self-assembling kinetics of the two disulphides may be different, the actual fraction of active groups on the surface cannot be presumed to be equal to the ratio of the two disulphides in solution. The surface concentration of hemin molecules, that is, the distance between adjacent hemin molecules, did not have any influence on the peak separation of the registered cyclic voltammograms (ΔE_p), but a slight increase (10–20 mV) in $E^{\circ\prime}$ was observed at low concentrations of the activated disulphide. This finding appears to indicate that with increasing surface coverage, interactions between adjacent hemin moieties may occur that in turn may affect $E^{\circ\prime}$.[33]

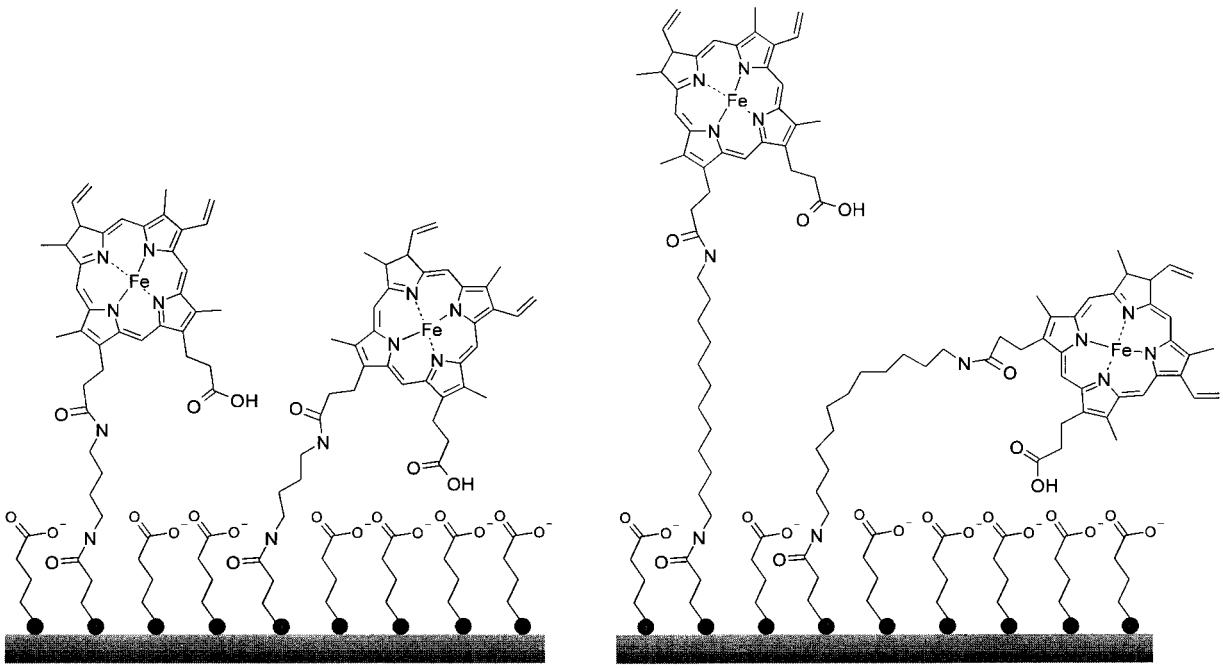

Figure 4. Schematic representation of the quasidiffusional variation of the ET distance for hemin-modified electrodes with different spacer lengths (C4 and C12).

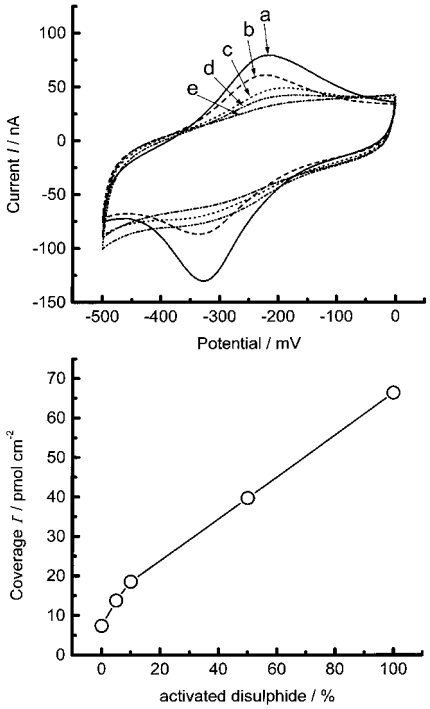

Figure 5. Top: cyclic voltammograms of hemin-modified electrodes with 1,12-diaminododecane as spacer at different ratios of activated disulphide and carboxylated disulphide (a = 100%; b = 50%, c = 10%, d = 5%; e = 0%). Scan rate 50 mV s^{-1}. Bottom: surface coverage of hemin calculated from the corresponding peak areas (a–e) of the cyclic voltammograms plotted as a function of the dilution of the activated headgroups in the monolayer.

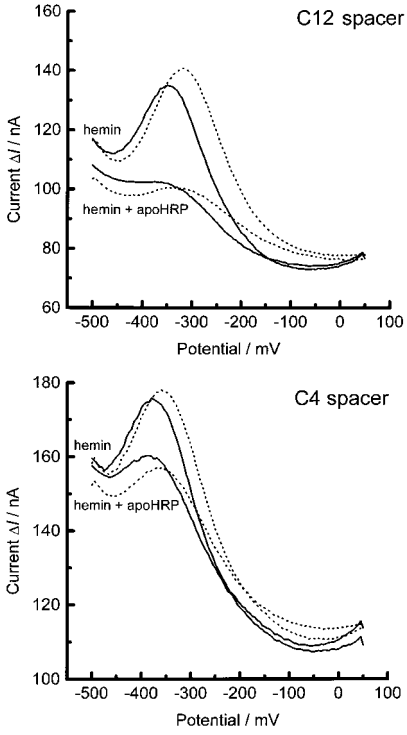

Figure 6. Differential pulse voltammograms: C12-spacer hemin electrode (top) and C4-spacer hemin electrode (bottom) before and after treatment with apo-HRP. (Scan rate 10 mV s^{-1}, 10% activated disulphide; solid line: oxidative scan, dashed line: reductive scan.)

Reconstitution of HRP on hemin-modified monolayers: As stated above, the distance from the carboxylic acid groups of the heme to the surface of the protein in HRP is at least 10 Å. The distances between the two nitrogen atoms in the diaminoalkanes used (straight conformation of the molecule) is estimated to be approximately 6, 9 and 16 Å for the diaminoalkanes with 4, 6 and 12 carbon atoms, respectively. Hence we assume that hemin bound to a 1,12-diaminododecane (C12-spacer) monolayer will reach into the active site of HRP (note that the disulphide used to dilute the activated disulphide is one carbon–carbon bond longer than the activated disulphide). The length of 1,6-diaminohexane (C6-spacer) is thought to be at the borderline, while 1,4-diaminobutane (C4-spacer) is most probably too short.

The reconstitution of holo-HRP is achieved by treating the hemin-modified electrode with a solution containing apo-HRP. A range of electrodes differing with respect to hemin surface concentration and the length of the spacer chain have been evaluated.

After treatment of the hemin electrodes with apo-HRP solution, the hemin redox waves in cyclic and differential pulse voltammetry are significantly diminished in the case of the C12-spacer (Figure 6, top). Since the shape and height of the voltammograms are not only modulated by the concentration of the redox-active species but also by the ET kinetics, it is possible that the redox waves observed after reconstitution of the holo-HRP are indicative for a reconstituted enzyme that has slower ET kinetics due to an increased ET distance. However, even on the assumption that the redox wave at -350 mV, after reconstitution of holo-HRP, is in the worst case still indicative of the free hemin redox reaction, one can derive that a maximum of about 24% of the hemin groups are in direct electrochemical communication with the electrode surface in the case of the longest spacer. This derivation is based on the average of the charges transferred during the anodic and cathodic wave of the differential pulse voltammogram. Evidently, after HRP reconstitution most surface-bound hemin groups are tightly bound within the active site of the protein; this prevents their whip-like approach to the monolayer surface. Therefore, the average overall ET distance between the hemin centre and the electrode surface increases significantly upon reconstitution. This finding is in good agreement with the observations reported for FAD-modified electrodes after reconstitution of glucose oxidase[25] and for hemin-modified electrodes after reconstitution of myoglobin.[28] Control experiments with holo-HRP and bovine serum albumin (BSA), as a means to investigate the effect of nonspecific adsorption on the electrode surfaces, showed only little influence on the hemin redox waves.

In contrast, after the attempt to reconstitute holo-HRP on the C4-spacer monolayer, the hemin redox waves were preserved to a value of about 57% of the transferred charge (Figure 6, bottom). This finding strongly suggests that a proper reconstitution of holo-HRP with the short-spacer bound hemin is unlikely. Owing to the small electrode surface area used in the experiments, a direct determination of the surface coverage of the electrodes with reconstituted HRP was not possible. Preliminary attempts to evaluate the surface coverage of reconstituted HRP by means of an electro-

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chemical quartz crystal micro balance (EQCM) did not succeed because of the extremely small amount of hemin binding sites in the 1:99 diluted thiol monolayers. Although optical methods such as ellipsometry or surface plasmon resonance might help to determine the surface coverage, these methods also detect inactive proteins and are, as a consequence, not appropriate to quantify the number of biologically intact reconstituted HRPs on the electrode surface. However, as shown below, the reconstitution of HRP in its bioactive form could be demonstrated by using a standard amperometric assay in the presence of an artificial electron donor.

Constant-potential amperometry in the presence of increasing concentrations of H_2O_2 demonstrated the possibility of direct ET between reconstituted HRP and the electrode. The reduction of H_2O_2 starts at potentials as high as 400 mV; this is indicative of the formation of the oxidised HRP intermediate [compound I, Fe^{IV}=O, P$^{\bullet}$ (porphyrin radical)] (Figure 7).

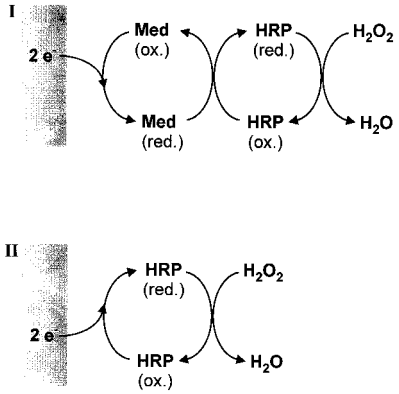

Figure 7. Reaction sequence of the HRP-catalysed reduction of H_2O_2 involving direct and mediated electron transfer between the enzyme and the electrode surface.

To prove that the observed electrocatalytic current is not caused by free hemin groups at the surface, direct and *ortho*-phenylenediamine-mediated ET were compared by use of a working potential of -100 mV (Figure 8). *Ortho*-phenylenediamine was shown to be inactive with hemin, whereas it is a very efficient electron donor for HRP in solution (with a second-order rate constant of $10^7-10^8\,M^{-1}s^{-1}$ [34]) as well as for HRP adsorbed on an electrode surface.[35] Recently, it was proposed that artificial phenolic electron donors (including such compounds as *ortho*-phenylenediamine) interact with oxidised HRP at an amino acid residue (Phe 179) in the vicinity of the active site.[36] This would explain why only oxidised HRP, and not oxidised hemin, undergoes a redox reaction with *ortho*-phenylenediamine. For short spacer chains (which are supposed to be too short to allow proper reconstitution of holo-HRP) the current obtained in the absence and presence of the free-diffusing donor was identical (Figure 8, top), while a significant increase in the current was observed for the longer spacer chain (Figure 8, bottom). These findings prove the successful reconstitution of the active holo-HRP in the case of the longest spacer (C12). However, evidently the distance for direct ET is too long to allow fast and efficient electrical communication between

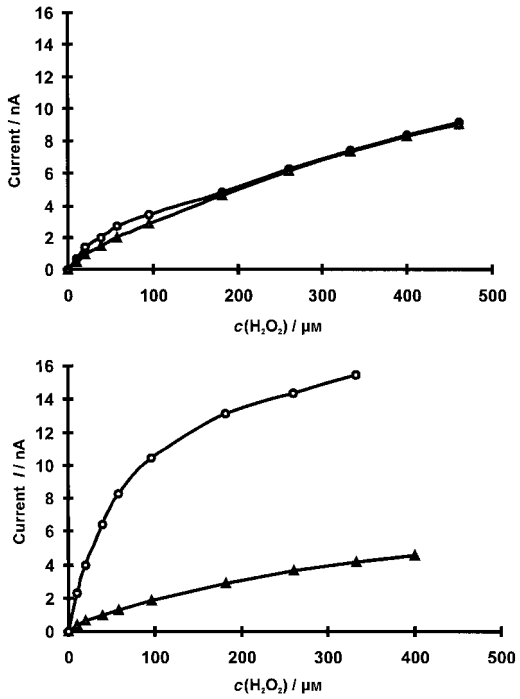

Figure 8. Comparison of electrocatalytic currents at hemin-modified electrodes after treatment with apo-HRP. Top: C4-spacer in the absence (▲) and in the presence of 2 mM *o*-phenylenediamine (○) (mediated ET). Bottom: C12-spacer in the absence (▲) of an electron donor (direct ET) and in the presence of 2 mM *o*-phenylenediamine (○) (mediated ET) (applied potential: -100 mV).

HRP and the electrode via the monolayer. Taking into account the significant decrease of the hemin redox wave to about 24% of its initial charge after reconstitution of holo-HRP on the C12-spacer surface, one would only expect a contribution of approximately 24% of free hemin sites to the overall reduction current observed with this electrode. As the currents are similar at -100 mV for the electrocatalytic reduction on the C12-hemin electrode and for the electrode on which HRP has been reconstituted, the predominant contribution to the electrocatalytic reduction current is probably due to a direct ET process from the active site of the properly orientated holo-HRP on the monolayer surface.

On the other hand, in the case of the C4 and C6 spacers, the reconstitution was obviously not successful leaving free hemin groups on the surface which caused the observed electrocatalytic current for the reduction of H_2O_2.

To support the argument concerning direct ET between reconstituted HRP and the electrode surface, one has to discuss the effect of the ET distance on ET kinetics. The electrochemistry of hemin, revealed by cyclic voltammetry, almost disappears after reconstitution of holo-HRP on the C12-hemin-modified electrodes, whereas the electrocatalytic current for H_2O_2 measured at a stationary potential of -100 mV remains virtually the same as for the hemin-modified electrode. The parameters of direct ET depend drastically on interfacial interactions and on the structure of the biologically active layers on electrodes. A true monolayer coverage of the electrode surface is especially important, even in the case of the maximally exposed catalytic centre. Not being shielded by a polypeptide chain, "naked" heme exhibits

heterogeneous ET rates higher than 4000 s^{-1} ($Fe^{3+/2+}$ redox conversion) when adsorbed on basal pyrolytic graphite or glassy carbon.[37, 38] High heterogeneous ET rates can be observed for heme adsorbed on gold electrodes modified with short thiols like ethanethiol, cystamine and mercaptoethanol with a resulting apparent heterogeneous ET rate constant of up to 3600 s^{-1}.[39] The highest ET rate constant reported so far for microperoxidase-11 (MP-11) on cystamine-modified gold electrodes was between 12[20] and 20 s^{-1}.[21] Most likely, a true monolayer of MP-11 was not present on these electrode surfaces; this would explain the comparatively low ET rate constants. On the other hand, by linking MP-11 thorugh its amino functions to the carboxyl-terminated short thiol monolayers, ET rate constants up to 1700 – 3600 s^{-1} were found.[22]

Several important and perhaps general conclusions can be derived by comparison of the vast number of heterogeneous ET measurements done with cytochrome *c* on thiol-monolayer-modified gold electrodes bearing carboxylic acid headgroups. It appears that in the case of long thiol modifiers (longer than 7 – 9 methylene groups) the interfacial ET drops exponentially with the length of the spacer. Evidently, the rate of electron tunnelling through the thiol layer is rate limiting in this case.[40, 41] However, no distance dependence was observed for monolayers formed from shorter thiol compounds, for which experimentally determined ET rate constants varied between 400 and 2000 s^{-1}. Remarkably, after covalent binding of cytochrome *c* to a thiol-modified gold electrode, the ET rate decreases by at least two orders of magnitude.[42]

For HRP, a protein molecule that is even larger than cytochrome *c*, the heme moiety is deeply buried within the protein shell (approximately 10 Å). Moreover, plant peroxidases are heavily glycosylated. In a recent paper,[43] the direct ET rate constants for native HRP (glycosylated, MW 44 kDa) and its recombinant nonglycosylated counterpart (MW 34 kDa) have been found to be around 2 and 8 s^{-1}, respectively, when the enzymes were adsorbed on graphite electrodes. The value of the ET rate constant for native HRP agrees with that of other investigators.[44]

Considering the slow ET between graphite and adsorbed HRP, it is not surprising that the electrochemistry of hemin virtually disappears after its incorporation into holo-HRP. Nevertheless, a clear electrocatalytic current for H_2O_2 reduction is noticed, just as for graphite electrodes modified with adsorbed HRP.

This raises an important question: how are the electrons transferred from the electrode to the active site of oxidised, reconstituted HRP? It has been shown that the critical distance for tunnelling of electrons between a graphite electrode and HRP is less than 18 Å.[45] The estimated distance between the reconstituted HRP and the electrode in the case described in this work is at least 16 – 18 Å, which is probably at the critical limit for electron tunnelling. Recently, Willner and co-workers[26] clearly demonstrated that no bioelectrocatalytic current could be obtained when glucose oxidase was reconstituted on a gold electrode modified with a FAD – carbon-chain – thiol derivative of similar length as used in this work. However, similar to our observations, the electrochemistry of FAD totally disappeared after reconstitution of holo-glucose oxidase on the electrode surface. This points to the important conclusion that efficient electron tunnelling was not achieved along the carbon chain. Since very efficient ET could be obtained from reduced FAD in the active site of reconstituted glucose oxidase to the electrode[26] after introduction of either a soluble redox mediator (e.g., a ferrocene derivative) or a covalently bound spacer-integrated mediator (pyrroloquinoline quinone), tunnelling through the σ-bonds of the methylene spacer seems unlikely. According to this argument, it seems unlikely that the electrochemical communication between the gold electrode and oxidised, reconstituted HRP, in the presence of H_2O_2, occurs through tunnelling along the backbone of the spacer. Another tentative explanation is that electrons tunnel through the protein shell of the reconstituted holo-HRP. However, more investigations are needed to put forward evidence for either of these hypotheses.

Conclusion

A sequential electrode modification procedure has been developed that enables us to optimise the distance between an immobilised cofactor like hemin, as a site for reconstitution of a holo protein, and the electrode surface without having to synthesise a variety of cofactor-thiol derivatives. This method can in principal be transferred to other cofactors to generate properly orientated holo proteins on monolayer surfaces in order to improve the direct electron transfer between the redox protein and the electrode surface. It was demonstrated that active HRP can be reconstituted on a surface that separated the hemin and the monolayer surface by a long spacer chain (C12), while no productive reconstitution was observed for shorter chains. However, the ET distance from the active site of the reconstituted HRP to the monolayer and electrode surface is rather large, thus causing slow ET kinetics. Future work will aim at decreasing the ET distance by the integration of suitable redox relays into the spacer chains.

Experimental Section

Chemicals: Horseradish peroxidase (HRP; RZ = 3.4) was purchased from Biozyme (Gwent, UK). 1,4-Diaminobutane, 1,12-diaminododecane and 3,3′-dithiodipropionic acid di-(*N*-succinimidyl ester) were obtained from Fluka (Buchs, Switzerland); 1,6-diaminohexane from Merck (Darmstadt, Germany); 3-carboxypropyl disulphide and cystamine-dihydrochloride from ACROS Organics (Geel, Belgium); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and hemin from Sigma (Deisenhofen, Germany); and *ortho*-phenylenediamine from Merck-Schuchardt (Hohenbrunn, Germany). Butanone, DMSO and ethanol were purchased from Riedel de Haen (Seelze, Germany). The buffer salts ($K_2HPO_4 \cdot 3H_2O$ and KH_2PO_4) were analytical grade and obtained from Merck (Darmstadt, Germany). H_2O_2 (30 %) was obtained from Baker (Deventer, Netherlands).

Preparation of apo-HRP: The heme of HRP was extracted following a method based on the work of Teale.[29] HRP was dissolved in cold phosphate buffer (100 mM) at pH 2.4 to yield a final concentration of 1 mg mL^{-1}. This solution (1 mL) was added to a separation funnel containing ice-cold butanone (1 mL). After mixing, the phases were allowed to separate, resulting in a aqueous phase containing the apo-HRP and a butanone phase containing the extracted heme. The aqueous phase was collected and the pH of this phase was quickly adjusted to pH 7 – 8. The

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resulting apo-HRP solution contains a high degree of dissolved butanone, which may be removed by dialysis or size-exclusion chromatography. However, for reconstitution experiments no further purification was made. The apo-HRP solution was diluted with water to obtain a final concentration of 0.1–0.2 mg mL^{-1} apo-HRP in phosphate buffer (10 mM).

Electrode preparation: Gold disk electrodes sealed in Kel-F (purchased from CH-Instruments, Cordova, TN, USA, with a surface area of 0.0330 cm^2) were cleaned by electrochemical cycling, in degassed NaOH (0.2 M), with a scan rate of 50 mV s^{-1} between -1000 mV and -1800 mV relative to Ag/AgCl (8 scans). The cleaned electrodes were immersed into a solution of DMSO containing an overall thiol concentration of 100 mM, while the ratio of activated and nonactivated thiocarboxylic acids varied between 0 and 100%. After an incubation time of 90 min at room temperature, the electrodes were thoroughly rinsed with water and immediately transferred to a diaminoalkane solution (20 mM; 1,6-diaminohexane, 1,4-diaminobutane or 1,12-diaminododecane) in water (or 95% ethanol for 1,12-diaminododecane). After 4 h the electrodes were carefully rinsed with water and transferred into the hemin coupling solution. For this, hemin was dissolved in DMSO to a final concentration of 1.0 mM and then diluted ten times using HEPES buffer (10 mM, pH 7.8) containing EDC (150 mM). The hemin coupling was allowed to proceed at 4 °C overnight. After careful rinsing with water the electrodes were immersed in $NaClO_4$ solution (100 mM) in DMSO in order to remove hemin that was merely adsorbed from the electrode surface. Finally, the electrodes were incubated with apo-HRP solution (prepared as described above containing 0.1–0.2 mg mL^{-1} apo enzyme) for 3–4 h at 4 °C.

Electrochemical measurements: Electrochemical measurements were performed with a potentiostat (EG&G 263A, Princeton Applied Research, Bad Wildbad, Germany), an Ag/AgCl reference electrode, and a platinum wire counter electrode. Phosphate buffer (20 mM, pH 7.0), which was carefully degassed with deoxygenated argon prior to the experiments, was used as the electrolyte. To maintain the inert atmosphere argon was passed over the solution during the measurements. In cyclic voltammetry three scans were recorded and the third scan was always used for comparison of different electrodes and evaluation of the surface coverage. For differential pulse voltammetry the following parameters were used: scan rate, 10 mV s^{-1}; pulse amplitude, 50 mV; pulse duration, 100 ms and step time, 500 ms. All potentials are given with respect to the Ag/AgCl/3 M Cl^- reference electrode.

Peroxidase activity measurements: The direct ET properties of reconstituted HRP-modified electrodes were determined by constant-potential amperometry at a potential of -100 mV relative to Ag/AgCl in phosphate buffer (20 mM) by means of the electrocatalytic reduction of H_2O_2 in the absence of any artificial electron donor. The steady-state current after successive addition of aliquots of H_2O_2 solution was plotted against the H_2O_2 concentration. For evaluation of the overall HRP activity of the electrode *ortho*-phenylenediamine (2 mM initially) was added to the electrolyte solution.

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