# Electrochemically and Catalytically Active Reconstituted Horseradish Peroxidase with Ferrocene-Modified Hemin and an Artificial Binding Site

**Alexander D. Ryabov,*[a] Vasily N. Goral,[a] Lo Gorton,[b] and Elisabeth Csöregi[c]**

**Abstract:** A procedure for modification of hemin chloride by $FcCH_2NH_2$ (Fc = ferrocenyl) in the presence of 1-(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and *N*-hydroxysuccinimide affords two main products **1** and **2** with mono- and bis-amidated propionic acid residues. Monoamidated conjugate **1** was loaded into the apoenzyme of horseradish peroxidase (HRP) to afford an electrochemically and catalytically active reconstituted enzyme Fc-HRP with remarkably altered substrate specificity. With ABTS as substrate, the reactivity of Fc-HRP drops threefold compared with native HRP as a result of a lowering of the maximal rate $V_m$. Compared with HRP the reactivity of Fc-HRP towards water-soluble ferrocenes is even higher at low concentrations of the latter, the rate increase being accompanied by a change in rate law: in contrast to first-order kinetics in ferrocenes for native HRP, there is a Michaelis dependence for Fc-HRP. Molecular modeling suggests creation of an artificial hydrophobic binding site within a triangle confined by the ferrocenyl residue and the two phenyl rings of Phe 68 and 179. The site is believed to be responsible for the kinetically meaningful binding between ferrocene substrates and Fc-HRP which manifests in the saturation kinetics.

**Keywords:** electrochemistry · enzyme catalysis · heme proteins · peroxidases · sandwich complexes

## Introduction

The importance of introducing transition-metal species into oxidoreductases for creation of heavy-metal and redox probes or artificial electron-transfer pathways is now well recognized and thoroughly accepted.[1–7] There are different ways to achieve this goal, and major progress has been achieved as a result of the covalent attachment of ferrocene derivatives to flavin adenine dinucleotide (FAD) of glucose oxidase (GO)[8] and a specific modification of a cytochrome $P450_{cam}$ mutant with cysteine residues exposed at the enzyme surface.[9] These studies were preceded by several less specifically targeted modifications of the surface functional groups of GO[10–16] and horseradish peroxidase (HRP).[17] Naturally, the wiring of redox centers directly to the active sites of redox enzymes, namely, FAD and hemin in the case of GO and HRP, respectively, is more advantageous than random coupling with surface amino acid residues. However, such a procedure is not easily accomplished in the case of FAD,[18] whereas the carboxylic groups of the propionic acid residues of hemin seem to be perfect candidates for binding to metal species. However, some doubt was evoked by an earlier work by Tamura et al., in which it was stated that reconstituted HRP with hemin monomethyl ester possesses only a 20% residual activity,[19] indicative of the fact that modifying agents bulkier than methyl could cause an even greater decrease in the enzymatic activity. On the other hand, our recent mechanistic work[20, 21] has demonstrated that alkylferrocenes are reactive substrates of HRP that do not inactivate the enzyme, and has suggested firstly that their location in the proximity of the hemin may not diminish the enzymatic activity strongly, secondly that the modified enzyme could be electrochemically active, and thirdly that the reconstituted biocatalyst may demonstrate novel, unexpected catalytic behavior. Several of these expectations proved true, and these constitute the subject of this report. Here, a procedure for monoamidation of hemin by aminomethylferrocene to afford **1** is presented, along with its loading into apo-HRP (HRP without its cofactor) and results on the electrochemical properties and catalytic activity of the reconstituted enzyme towards water-soluble ferrocenes and ABTS.

[a] Prof. A. D. Ryabov, V. N Goral
Department of Chemistry, Moscow State University
119899 Moscow (Russia)
Fax: (+7) 095-939-5417
E-mail: ryabov@enzyme.chem.msu.su

[b] Prof. L. Gorton
Department of Analytical Chemistry
Center for Chemistry and Chemical Engineering, Lund University
P.O. Box 124, S-221 00 Lund (Sweden)

[c] Dr. E. Csöregi
Department of Biotechnology
Center for Chemistry and Chemical Engineering, Lund University
P.O. Box 124, S-221 00 Lund (Sweden)

  0947-6539/99/0503-0961 $ 17.50+.50/0 

## Experimental Section

**Materials and methods**: The reagents tris(hydroxymethyl)aminomethane, $H_2O_2$, $K_2HPO_4$, $KH_2PO_4$, HCl (all highest grade) were purchased from Reakhim (Russia). FcCOOH was a Fluka reagent; $FcCH_2NMe_2$ was kindly provided by Dr. M. D. Reshetova (Moscow State University). ABTS was a Sigma reagent. Spectrophotometric measurements were carried out on a Shimadzu UV-160A spectrophotometer equipped with a CPS-240A cell positioner/temperature controller. Cyclic voltammograms were obtained on a PC-interfaced potentiostat-galvanostat IPC-3 (Institute of Physical Chemistry, RAS, Moscow, Russia). A three-electrode scheme was used with working pyrolytic graphite electrode, saturated calomel reference electrode (SCE), and auxiliary Pt electrode. All potentials reported in this paper are given against SCE. The electrochemical cell was kept at 25 °C by circulating water. Infrared spectra were recorded on a Bio-Rad FTS-6000 spectrophotometer in KBr disks. Fast atom bombardment mass spectra were obtained on a Jeol SX-102 spectrometer. Isoelectrofocusing experiments were performed with a BioRad Model 111 instrument.

**Preparation of conjugates**: Aminomethylferrocene (64.6 mg, 0.30 mmol), which was prepared from ferrocene carboxylic acid (Aldrich) as described elsewhere,[22] and hemin chloride (ICN, 197 mg, 0.30 mmol) were each dissolved in 20 mL dimethylformamide and then mixed. 1-(Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (ICN, 87.6 mg, 0.46 mmol) and *N*-hydroxysuccinimide (ICN, 52 mg, 0.45 mmol) were added with stirring, and the solution was kept at room temperature for 10 h. When the amine was consumed (monitored with the ninhydrin test after TLC on a silica gel alumina plate), the mixture was filtered, and aqueous HCl (ca. 110 mL, pH 1–2) was added to induce the formation of a brown precipitate. The latter was filtered off, washed with a large amount of water to neutral according to Merck pH indicator strips, and dried under vacuum over $P_2O_5$. The dry material was dissolved in a minimal amount of $CHCl_3$ and the products formed were separated by column chromatography ($12 \times 4.5$ cm) on silica gel (Merck, 0.063–0.200 mm). The products were eluted with 10:1 and then 4:1 $CHCl_3$/MeOH mixtures. Disubstituted hemin derivative **2** was eluted first followed by the monosubstituted product **1**. Evaporation of solvent in vacuum gave 45 and 19 mg of **2** and **1**, respectively, as brown solids. $R_f$ (silica gel plates, $CHCl_3$/MeOH = 6:1): 0.6 and 0.4; $FAB^+$: 1010 and 810 for **1** and **2**, respectively ($m/z - Cl^-$). The samples were free from starting hemin chloride; this was suggested by the lack of the peak at ($m/z - Cl^-$) 616. The more hydrophobic **2** is soluble in common organic solvents (chloroform, benzene, MeOH, DMSO), sparingly soluble in *n*-hexane, and can be recrystallized from $CHCl_3/C_6H_{14}$. Monosubstituted derivative **1** is sparingly soluble in MeOH and less soluble in other organic solvents. It was noted that its solubility diminished on aging, most likely due to the formation of a less soluble oxo-bridged dimer.[23–25]

Модификация хлорида гемина под действием $FcCH_2NH_2$ (Fc = ферроценил) в присутствии 1-(диметиламинопропил)-3-этилкарбодиимидгидрохлорида и N-гидроксисукцинимида приводит к двум продуктам **1** и **2** с моно- и диамидированными пропионовокислыми остатками соответственно. Встраивание моноамидированного конъюгата **1** в апо-форму пероксидазы из корней хрена приводит к электрохимически и каталитически активному реконструированному ферменту Fc-HRP с измененной субстратной специфичностью. При использовании ABTS в качестве субстрата реакционная способность Fc-HRP уменьшается в три раза по сравнению с нативной HRP вследствие снижения максимальной скорости $V_m$. Реакционная способность Fc-HRP по отношению к водорастворимым ферроценам даже выше при низких концентрациях последних, причем увеличение скорости сопровождается изменением кинетического уравнения реакции. В отличие от кинетики реакции первого порядка по ферроценам для нативного фермента в случае Fc-HRP наблюдается михаэлисовская зависимость. Молекулярное моделирование реконструированнго белка свидетельствует о создании искусственного гидрофобного центра связывания, ограниченного треугольником, в вершинах которого расположены ферроценовое ядро и два фенильных кольца остатков Phe 68 и 179. Сделан вывод о том, что именно этот центр несет ответственность за кинетически значимое связывание между ферроценовыми субстратами и Fc-HRP, которое проявляется в кинетическом "насыщении" по ферроценам.

**Reconstitution of HRP**: Apo-HRP was prepared as described elsewhere[26] from HRP isoenzyme C which was a Dia-M product (Russia, $RZ = 3.2$, ammonium sulfate precipitate). HRP (15.4 mg) was dissolved in KCl (3 mL, 0.1 M) and the pH of the ice-cold solution was adjusted to 1.9 by addition of HCl (0.1 M). An equal volume of ice-cold methyl ethyl ketone was added three times to this solution and the mixture was shaken for ca. 10 s. The solution was kept at 0 °C for separation and the lower aqueous layer containing apo-HRP was passed through a Sephadex G 25 superfine column ($21 \times 1.7$ cm) to remove the ketone and other low-molecular-weight impurities. The UV-Vis spectrum of the apoprotein showed that hemin was removed from HRP, since there was no absorbance at 403 nm typical of hemin in the active center of HRP. The amount of apo-HRP after gel filtration determined by the Lowry method[27] (bicinchoninic acid protein assay kit, Sigma) equals 10.3 mg in Tris/HCl buffer (7.5 mL). Conjugate **1** (0.45 mg, $5.4 \times 10^{-4}$ mmol) was dissolved in dimethyl sulfoxide (400 μL), and 100 μL of the solution was added to phosphate buffer (900 μL, pH 7.0) to afford a mixture with concentrations of **1** and DMSO of $1.35 \times 10^{-4}$ and 1.4 M, respectively. The solution of **1** (114 μL, a 150% excess with respect to apo-HRP) was added dropwise with stirring to the solution of apo-HRP (5 mL, 6.86 mg) at 0 °C over 30 min. The resulting solution of reconstituted HRP was passed through a column of Sephadex G 25 Superfine to separate the enzyme from the excess of unbound **1** and DMSO. The amount of reconstituted protein in solution after gel filtration was determined by the Lowry method ($9.76 \times 10^{-4}$ g mL$^{-1}$, $1.3 \times 10^{-5}$ M).

**Extraction of 1 from reconstituted HRP**: To ensure that the reconstituted Fc-HRP contained **1**, the following experiment was performed. The pH of 1 mL solution of Fc-HRP was adjusted to 2 by addition of HCl (0.1 M) at 0 °C. Conjugate **1** was extracted into methyl ethyl ketone (300 μL), 100 μL of this solution were mixed with acetonitrile (1 mL) containing $n$Bu$_4$NPF$_6$, and the resulting solution was investigated by cyclic voltammetry.

**Kinetic measurements with Fc-HRP**: Kinetic measurements with reconstituted Fc-HRP were conducted by the example of water-soluble ferrocene derivatives FcCOOH and $FcCH_2NMe_2$ as described previously.[21] Solutions of FcCOOH and $FcCH_2NMe_2$ were prepared in phosphate buffer (0.013 M, pH 7.0) and their concentrations were checked spectrophotometrically. The corresponding extinction coefficients $\varepsilon$ ($\lambda_{max}$, nm) for FcCOOH and $FcCH_2NMe_2$ equal 238 (439) and 150 (428) M$^{-1}$cm$^{-1}$, respectively. Concentrations of stock solutions of FcCOOH and $FcCH_2NMe_2$ were $2.3 \times 10^{-3}$ and $4.6 \times 10^{-3}$ M, respectively. Solutions of $H_2O_2$ were standardized by UV-Vis spectroscopy with an extinction coefficient of 72.8 M$^{-1}$cm$^{-1}$ at 230 nm.[28] The reactions were initiated by the addition of HRP (15 μL) or Fc-HRP solution ($1.34 \times 10^{-5}$ M) to the reaction mixture containing a water-soluble ferrocene derivative (1.0 mL, $2.3 \times 10^{-3}$ M), hydrogen peroxide (30 μL, $1.4 \times 10^{-2}$ M), and phosphate buffer (955 μL, 0.013 M, pH 7) in a 1 cm quartz cuvette. The development of absorbance was registered at the wavelength of maximum absorption of products.

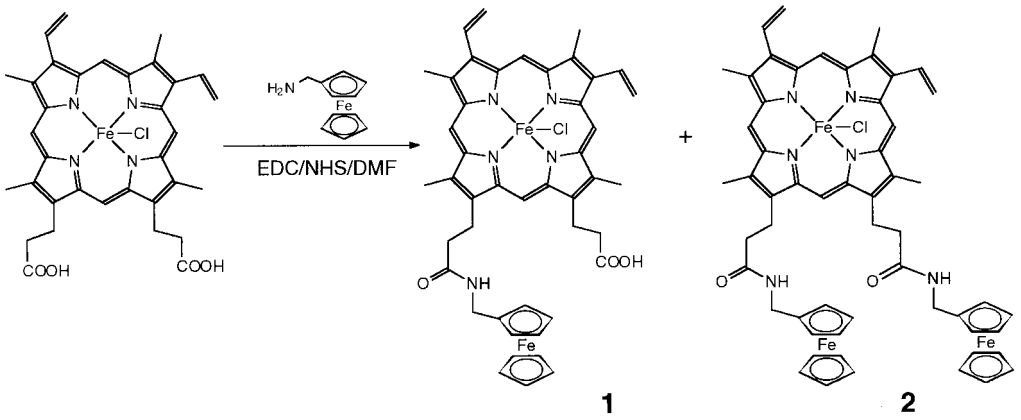
**Structural modeling of Fc-HRP**: The modeling of the reconstituted enzyme Fc-HRP was carried out using the recently reported X-ray structural data of HRP.[29] The molecular structure of Fc-HRP was built using an Insight II (Biosym/Molecular Simulations) program on a Silicon Graphics workstation. The structure was partially optimized by a Discover 3.0 module by means of the ESFF molecular forcefield.

## Results

**Preparation and characterization of ferrocene conjugates 1 and 2**: The coupling between hemin chloride and aminomethylferrocene in the presence of 1-(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in dimethylformamide leads to the formation of mono- (**1**) and diamide (**2**), the latter being the dominant product (Scheme 1). Conjugates **1** and **2** can be separated by column and/or thin-layer preparative chromatography with silica gel and chloroform/methanol mixtures. As expected, the more hydrophobic **2** is more soluble in



0947-6539/99/0503-0962 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 3




Scheme 1. Formation of conjugates **1** and **2** from hemin chloride.

organic solvents than **1**, which is sparingly soluble in methanol and DMSO. The solubility of **1** increases in the presence of trace amounts of acetic acid. The composition of conjugates **1** and **2** was proved by $FAB^+$ mass spectrometry which gave information on dechlorinated hemins. It should be pointed out that attempts to apply the recently reported procedure with PyBop [benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate] as a coupling agent[30] gave less pure conjugates than the EDC synthesis.

The spectral changes of the infrared spectra in the carbonyl region of hemin chloride and conjugates **1** and **2** are indicative of the modification of carboxylic groups of the propionic acid residues. In particular, a strong band at 1701 $cm^{-1}$ observed in the case of hemin chloride shifts to 1641 and 1642 $cm^{-1}$ on going to **2** and **1**, respectively, as anticipated when converting the carboxylic group into an amide. The UV/Vis spectra of hemin chloride and conjugates **1** and **2** obtained in buffered aqueous solution and acetonitrile differ as well. Interestingly, the blue shift of the Soret band of **1** is stronger than that of **2** at pH 7.0; the maxima (extinction coefficients $\varepsilon$, $M^{-1}cm^{-1}$) observed at 378 ($1.75 \times 10^4$) and 394 ($1.52 \times 10^4$) nm, respectively, should be compared with that for hemin chloride at 385 ($5.28 \times 10^4$) nm in 0.03 M Tris/HCl, pH 8.0.

The presence of the ferrocene residues in **1** and **2** was supported by cyclic voltammetry measurements both in MeCN and aqueous solutions. The response from the ferrocene moiety is more pronounced in acetonitrile. In particular, a nice redox reaction in the case of **1** is characterized by the formal redox potential, $E^{\circ\prime}$, at 392 mV (Figure 1). The characteristics of **2** are similar and its cyclic voltammogram is characterized by a $E^{\circ\prime}$ value of 398 mV (not shown). The two ferrocene residues do not affect each other, as anticipated for a molecule with widely separated redox centers. Cyclic voltammograms of **1** and **2** in buffered aqueous solution at pH 7.0 also contain signals from the ferrocene fragments together with strong traces in the range from $-0.5$ to $-0.3$ V, the shape of which is typical of the species involved in a *EC′* electrochemical process[31] presumably of a hemin-catalyzed reduction of $O_2$ into water.[32] This behavior, however, differs from observations made previously by other workers in the case of hemin chloride. It was reported that a reversible electrochemical behavior is typical of the latter.[33, 34] Attempts to reproduce such voltammograms both by testing hemin chloride and bubbling nitrogen or argon through the aqueous solution failed. In fact, a decrease in the catalytic current was observed, but cyclic voltammograms unaffected by catalysis have not been obtained. However, it should be noted that it usually takes up to two days of continuous bubbling of nitrogen or argon through aqueous solutions to exclude the effect of oxygen completely.[35]

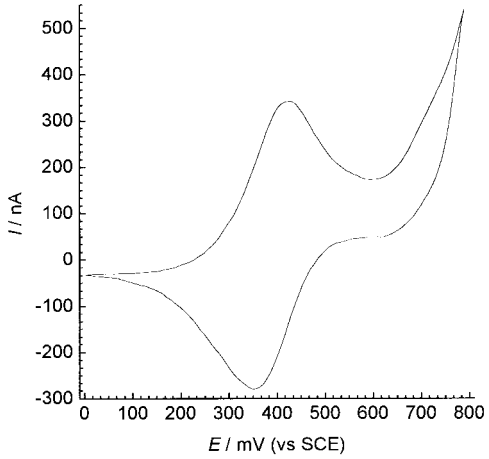



Figure 1. Cyclic voltammogram of **1** after subtraction of background noise obtained in MeCN ([**1**] $5 \times 10^{-5}$ M, $nBu_4NPF_6$ 0.026 M, 25 °C, scan rate 80 mV $s^{-1}$).

**Reconstitution of apo-HRP with ferrocene-modified hemin chloride 1**: Apo-HRP was prepared according to an acidic methyl ethyl ketone procedure of Teale.[26] After hemin removal, the apoenzyme was purified by gel filtration on Sephadex G25 (fine or superfine). It has been confirmed by UV/Vis spectroscopy that the degree of hemin removal was greater than 98% (Figure 2). The reconstitution of HRP with **1** to afford Fc-HRP was carried out by adding a solution of **1** in dimethyl sulfoxide to apo-HRP dissolved in Tris/HCl buffer (pH 8) and keeping the mixture at 0 °C for 0.5 h. Finally, the mixture was subjected to gel filtration as above. The loading of **1** into apo-HRP is strongly supported by UV/Vis spectroscopy. As seen in Figure 2, the maximum position and band intensity of **1** change on loading. Attempted reconstitution of HRP by **2** with the goal of obtaining $Fc_2$-HRP was performed in the same way.

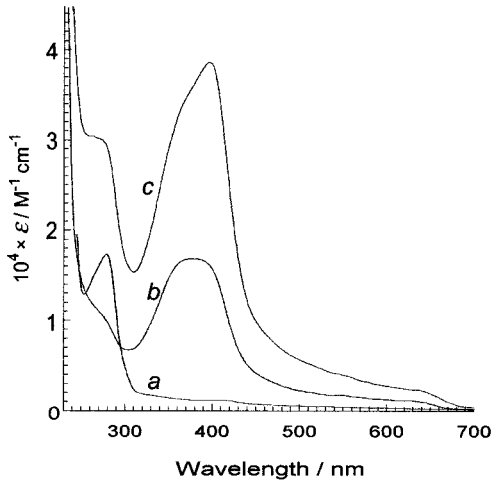



Figure 2. Electronic spectra of a) apo-HRP, b) **1**, and c) Fc-HRP in aqueous solution (25 °C, pH 8, Tris/HCl).

**Properties of Fc-HRP and $Fc_2$-HRP**: Preliminary experiments have shown that Fc-HRP, in contrast to $Fc_2$-HRP, is catalytically active (see below) and, therefore, this particular reconstituted peroxidase became an object of major research. The UV/Vis spectra of native HRP and Fc-HRP contained a first remarkable observation. The Soret bands were observed at 403 and 397 nm, respectively, pointing to a 6 nm shift on incorporation of **1** into apo-HRP (cf. the maximum position of **1** in aqueous solution). This indicates that a correct loading of **1** into apo-HRP has taken place and, when incorporated, modified hemin **1** attains spectral features typical of native hemin inside HRP. The *R*/*Z* ratio for the reconstituted enzyme was estimated as 1.3, and the relatively low value is attributed to the absorption of the ferrocene fragment at 280 nm (Figure 2). Isoelectrofocusing of Fc-HRP confirmed the homogeneity of the preparation, the isoelectric point of which is shifted, as could be anticipated, to a more basic pH (cf. 8.1 and 8.5 for HRP and Fc-HRP, respectively).

Cyclic voltammetry (CV) experiments indicated that the ferrocene moiety manifests itself even in Fc-HRP. Figure 3 shows voltammograms obtained for Fc-HRP and HRP, the latter for the sake of comparison. As seen, there are anodic and cathodic peaks around 360 and 300 mV in the case of Fc-HRP (b) which are absent in the case of native HRP (a). Subtraction of the HRP trace from that obtained for Fc-HRP gives a difference voltammogram of the ferrocene unit in the protein environment (c, inset) and allows location of the peak position. The estimated $E^{\circ\prime}$ of 327 mV is to be compared with that for **1** in aqueous solution (264 mV). The difference indicates that the ferrocene probe is shielded by amino acid residues located in the vicinity of the active site. These are likely to be the phenylalanine residues of the substrate access channel of HRP[29, 36] (see below).

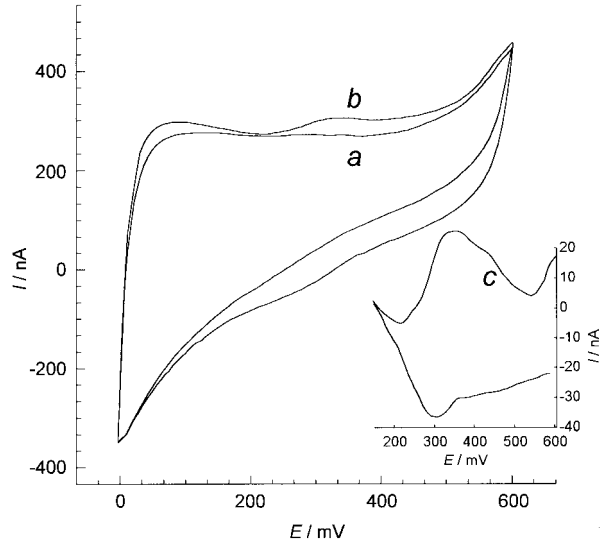



Figure 3. Cyclic voltammograms of a) HRP and b) Fc-HRP, and c) the difference (a – b) trace in aqueous solution ([Fc-HRP] $1.3 \times 10^{-5}$ M, scan rate 160 mV $s^{-1}$, 25 °C, pH 7). For details, see text.

It was possible to remove **1** from Fc-HRP by the procedure used for preparation of apo-HRP. This was done to confirm by CV that the ferrocene fragment dissociates from the enzyme together with hemin. The cyclic voltammogram recorded (not shown) is very similar to that shown in Figure 1 confirming the presence of the ferrocene unit in the sample. Therefore, it can be concluded that the linkage between ferrocene and hemin does occur in the reconstituted enzyme.

**Catalytic activity of reconstituted Fc-HRP**: It should be emphasized that it was only Fc-HRP that displayed any catalytic activity towards various peroxidase substrates; its counterpart $Fc_2$-HRP proved to be catalytically inactive. The Fc-HRP activity was quantitatively tested with respect to two different substrates of HRP, that is 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and water-soluble ferrocene derivatives. The former is a very common, extremely reactive substrate,[37] whereas the latter belong to a recently studied family of ferrocene substrates. These, rather unusually for fast enzymatic reactions, display first-order kinetics.[20, 21] Thus, it was a challenge to compare the rate laws and kinetic parameters, which determine the reactivity, of native HRP and Fc-HRP. The data in Figure 4 demonstrate

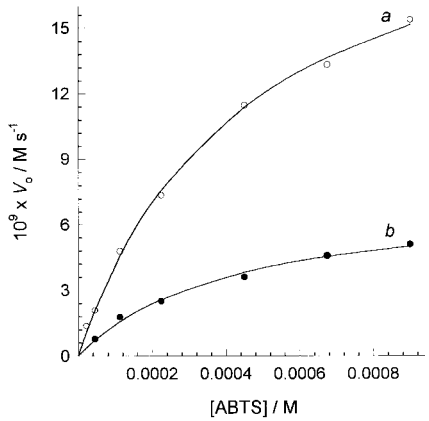



Figure 4. Steady-state rate of a) HRP- and b) Fc-HRP-catalyzed oxidation of ABTS by hydrogen peroxide. For conditions, see legend to Table 1.

that both the native and the reconstituted enzyme (Fc-HRP) catalyze the oxidation of ABTS in accordance with Michaelis kinetics. The $V_0^{-1}$ versus $[ABTS]^{-1}$ plots are linear and the values of the maximal rates $V_m$ and the Michaelis constant $K_m$ are summarized in Table 1. As seen, $K_m$ does not change, while $V_m$ decreases by a factor of three on going from HRP to



0947-6539/99/0503-0964 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 3

Table 1. Kinetic characteristics for native HRP and reconstituted Fc-HRP obtained at 25 °C (ABTS: pH 6.0 (0.1 M phosphate), $[HRP] = 1 \times 10^{-10}$ M, $[H_2O_2] = 7 \times 10^{-4}$ M; ferrocenes: pH 7.0 (0.13 M phosphate), $[HRP] = 1 \times 10^{-7}$ M, $[H_2O_2] = 2 \times 10^{-4}$ M).

| Substrate | $V_m$ [M s$^{-1}$] | $k_{cat} = V_m/[E]$ [s$^{-1}$] | $k_{cat}/K_m$ [M$^{-1}$ s$^{-1}$] | $K_m$ [M] |
|---|---|---|---|---|
| HRP (native) | | | | |
| ABTS | $(226 \pm 14) \times 10^{-10}$ | $226 \pm 14$ | $(51 \pm 5) \times 10^4$ | $(4.4 \pm 0.3) \times 10^{-4}$ |
| FcCOOH | – | – | $(0.89 \pm 0.03) \times 10^4$[a, b] | – |
| $FcCH_2NMe_2$ | – | – | $(0.24 \pm 0.01) \times 10^4$[a, b] | – |
| Fc-HRP | | | | |
| ABTS | $(71 \pm 4) \times 10^{-10}$ | $71 \pm 4$ | $(17 \pm 2) \times 10^4$ | $(4.2 \pm 0.5) \times 10^{-4}$ |
| FcCOOH | $(26 \pm 2) \times 10^{-7}$ | $26 \pm 2$ | $(2.4 \pm 0.3) \times 10^4$ | $(11 \pm 1) \times 10^{-4}$ |
| $FcCH_2NMe_2$ | $(4.03 \pm 0.06) \times 10^{-7}$ | $4.03 \pm 0.06$ | $(2.7 \pm 0.2) \times 10^4$ | $(1.4 \pm 0.1) \times 10^{-4}$ |

[a] From ref. [21]. [b] The second-order rate constant $k_3$ (rate $= k_3$[HRP][Fc]).

Fc-HRP. Hence, the overall loss in reactivity towards ABTS is as low as a factor of three.

In contrast to ABTS, the kinetic behavior of native HRP and Fc-HRP towards water-soluble ferrocenes, namely FcCOOH and $FcCH_2NMe_2$, is remarkably different. Instead of the strict first-order kinetics observed for native HRP,[20, 21] the reaction rate levels off on increasing the ferrocene concentration in the case of Fc-HRP (Figure 5). The Michaelis-type dependence is observed for both ferrocene substrates,

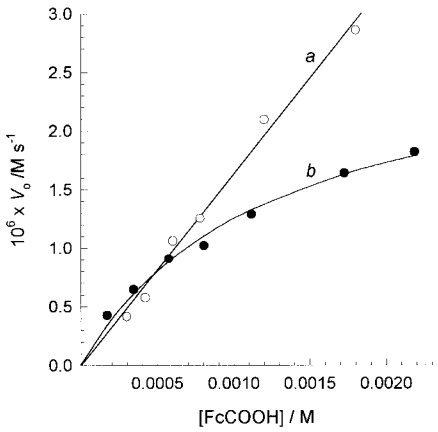



Figure 5. Steady-state rate of a) HRP- and b) Fc-HRP-catalyzed oxidation of FcCOOH by hydrogen peroxide. For conditions, see legend to Table 1.

and the $V_0^{-1}$ versus [ferrocene]$^{-1}$ plots are linear. The values of $V_m$ for FcCOOH and $FcCH_2NMe_2$, which are also summarized in Table 1 together with the corresponding values of $K_m$, differ by a factor of 6.4. Such a pronounced difference rules out a trivial rationalization of the Michaelis kinetics in catalysis by peroxidase and related enzymes which appears as a result of the rate-limiting interaction between the resting ferric state of the enzyme and hydrogen peroxide.[38] A higher (by a factor of 6.4) value of $V_m$ in the case of FcCOOH compared with $FcCH_2NMe_2$ is in accordance with a higher driving force of the reaction in the case of FcCOOH, the formal redox potential of which is lower than that of $FcCH_2NMe_2$ (290 and 370 mV versus SCE, respectively). It should also be noted that the reactivity of FcCOOH towards HRP and Fc-HRP is very close at low substrate concentrations (Figure 5). This prompts an intriguing comparison of the second-order rate constant $k_3$ and the $k_{cat}/K_m$ ratio in the case of HRP and Fc-HRP, respectively, which are summarized in Table 1. The conclusion is striking: the reactivity of the reconstituted enzyme towards artificial organometallic substrates (ferrocenes) is higher than that of the native enzyme in terms of the $k_3$ versus $k_{cat}/K_m$ formalism!

## Discussion

The modification of hemin by aminomethylferrocene affords two products **1** and **2**, the former being a mixture of two positional isomers difficult to separate. The chromatographic separation of the mono- and bis-amidated species described in this work and very recently by Monzani et al.[39] is obviously an alternative to the recently reported procedure involving amidation of protoporphyrin IX monoethyl ester by *N*,*N*-di(ethoxycarbonylmethyl)ethylenediamine ditrifluoroacetic acid salt followed by basic ester hydrolysis.[40] The loading of **1** into apo-HRP leads to a catalytically and electrochemically active biocatalyst with a remarkably modified activity towards certain electron donors. These features will be discussed further in the light of the recently reported X-ray structural data for HRP[29] and the results of our molecular modeling.

**Modeling of Fc-HRP**: The X-ray structural study of HRP revealed several interesting features of the enzyme, one of which is a fairly loosened arrangement of its active site.[29] Therefore, it was not very surprising to see that the positional isomer **1** could easily be embedded into the active site, Gln 176 being the only amino acid residue which was subjected to a significant alteration of its position. As a result, the ferrocene core is almost on the protein surface (Figure 6),

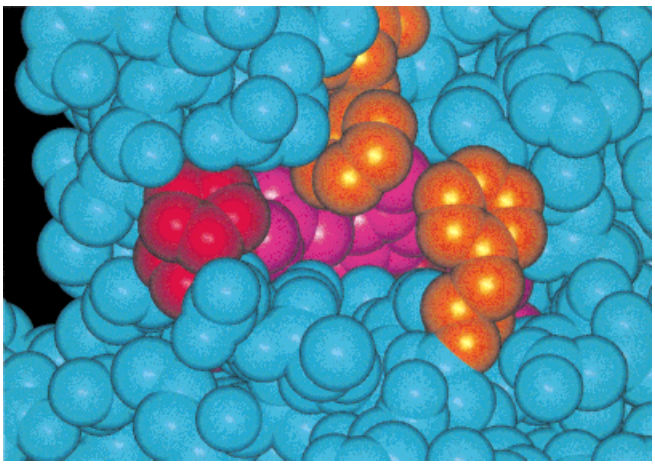


Figure 6. View of the Fc-HRP model showing that the ferrocene frame (in red) is located on the protein surface and the new artificial cavity within the triangle, the apexes of which are ferrocene and (shown in orange) Phe 179 (top) and 68 (right). Hemin atoms are colored in pink.

accounting for the electron exchange between the organometallic redox label and the electrode. It should be pointed out that similar results of structural modeling were obtained for another positional isomer, namely an analogue of **1** modified at position 18. In both cases the conclusions were similar, that is, both isomers could be easily incorporated into apo-HRP and the ferrocenyl fragment is always at the surface of HRP. Thus, in spite of the expected positional heterogeneity of Fc-HRP, both possible isoforms are structurally and presumably catalytically similar. It should only be indicated that the loading of the second positional isomer (i.e. **1**) into apo-HRP is slightly less sterically demanding.

As seen in Figure 6, there is a triangle with apexes made up of the ferrocenyl fragment and the phenyl rings of Phe 68 and 179. The critical importance of the latter two moieties for the binding of aromatic donor substrates of HRP has recently been emphasized on the basis of site-directed mutagenesis and $^1$H NMR studies.[36] All three apexes are hydrophobic, especially the Phe 68 residue. It seems reasonable to assume that such a spatial arrangement of the three groups creates a novel hydrophobic binding center on the enzyme surface, on one hand, and in the vicinity of hemin, on the other. If so, the novel binding site should perfectly correspond to such nonplanar aromatic substrates as ferrocenes. In fact, the distances between the $\eta^5$-$C_5H_5$ ring of ferrocene and the phenyl groups of Phe 179 and 68 fall in the range 4.6 – 6 and 6 – 8 Å, respectively. The dimensions of the new cavity thus assembled seem very advantageous for ferrocene binding. In our previous mechanistic work,[20] ferrocenes as HRP substrates were associated with ball-shaped species which for steric reasons were unable to approach close to hemin and to form kinetically relevant enzyme-substrate complexes. As a result, strict first-order kinetics in ferrocenes has been observed.

In contrast to the situation in native HRP, there is a novel artificial hydrophobic binding site at the surface of Fc-HRP created essentially by the ferrocenyl, Phe 68, and Phe 179 phenyl groups. Assuming that the structural similarity principle holds, that is structurally similar compounds possess enhanced affinity towards each other, the location of the ferrocenyl fragment in the novel binding site could drastically increase the binding ability of ferrocene substrates. The rate measurements have in fact demonstrated that the kinetic behavior of ferrocenes towards the reconstituted peroxidase has been significantly modified. Instead of the first-order kinetics in ferrocenes in the case of HRP, there is a Michaelis dependence for Fc-HRP, indicative of the kinetically relevant enzyme – substrate binding.

**Increased reactivity towards artificial substrates**: One may speculate that the reactivity of Fc-HRP towards ferrocenes in terms of the $k_{cat}/K_m$ formalism is higher than that of native HRP. As seen from Table 1, the ratio $k_{cat}/K_m$ exceeds the rate constant $k_3$ by a factor of 2.7 and 11 for FcOOH and $FcCH_2NMe_2$, respectively.[41] Naturally, such kinetic, model-dependent comparisons should be treated with care, since the estimates are valid only in a limited range of low substrate concentrations, that is when comparable formal rate laws are realized.

The Michaelis constant for ABTS does not change on going from HRP to Fc-HRP (see Table 1). This strongly suggests that the binding site for ABTS is virtually unaffected by introduction of the ferrocene residue. The $V_m$ value drops threefold on going from HRP to Fc-HRP, and this number looks very optimistic from the standpoint of possible modifications of the hemin propionic acid residues by molecules bulkier than the methyl group.

In conclusion, horseradish peroxidase reconstituted using hemin with a modified (by aminomethylferrocene) propionic acid residue retains its catalytic activity. In the case of ABTS, there is a 66 % decrease in the activity compared to native HRP. In the case of organometallic electron donors, specifically water-soluble ferrocenes, two simultaneous effects are observed, that is the change of rate law and an apparent increase in the catalytic activity. It is believed that the two are due to creation of a novel hydrophobic binding site at the enzyme surface for ferrocenelike molecules. It is located within a triangle confined by the ferrocenyl residue and the phenyl rings of Phe 68 and 179. This study opens up a wide horizon for modification of hemin propionic acid residues of various peroxidases and related enzymes by various species, including redox-active ones. Studies associated with electron-transfer processes from a wired ferrocene fragment (or any other suitable redox species) to the oxidized (by natural oxidants) hemin, and the efficacy of the electron relays electrode – ferrocene – hemin, are under way.

## Acknowledgments

The research described in this publication was made possible in part by financial support from the Russian Foundation for Basic Research (Grant No 96-03-34328a) and the Swedish Natural Science Research Council (NFR). The fellowship from the Wenner-Gren foundation for ADR is gratefully acknowledged. We thank Dr. T. Ruzgas for valuable, stimulating discussions and E. S. Ryabova for preliminary electrochemical measurements. ADR is very grateful to Prof. L.-I. Elding for the use of the facilities of Department of Inorganic Chemistry I, Lund University, for realization of the synthetic part of this work.

[1] A. E. G. Cass, G. Davis, G. D. Francis, H. A. O. Hill, W. J. Aston, I. J. Higgins, E. O. Plotkin, L. D. L. Scott, A. P. F. Turner, *Anal. Chem.* **1984**, *56*, 667 – 671.
[2] A. Heller, *Acc. Chem. Res.* **1990**, *23*, 128 – 134.
[3] A. Heller, *J. Phys. Chem.* **1992**, *96*, 3579 – 3587.
[4] A. D. Ryabov, *Angew. Chem.* **1991**, *103*, 945 – 955; *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 931 – 941.
[5] T. Ruzgas, E. Csöregi, J. Emnéus, L. Gorton, G. Marko-Varga, *Anal. Chim. Acta* **1996**, *330*, 123 – 138.
[6] I. Willner, E. Katz, B. Willner, *Electroanalysis* **1997**, *9*, 965 – 977.
[7] I. Willner, E. Katz, B. Willner, R. Blonder, V. Heleg-Shabtai, A. F. Bückmann, *Biosens. Bioelectron.* **1997**, *12*, 337 – 356.
[8] A. Riklin, E. Katz, I. Willner, A. Stocker, A. F. Bückmann, *Nature* **1995**, *376*, 672 – 675.
[9] K. Di Gleria, D. P. Nickerson, H. A. O. Hill, L.-L. Wong, V. Fülöp, *J. Am. Chem. Soc.* **1998**, *120*, 46 – 52.
[10] P. N. Bartlett, R. G. Whitaker, M. J. Green, J. Frew, *J. Chem. Soc. Chem. Commun.* **1987**, 1603 – 1604.
[11] Y. Degani, A. Heller, *J. Phys. Chem.* **1987**, *91*, 1285 – 1289.
[12] Y. Degani, A. Heller, *J. Am. Chem. Soc.* **1988**, *110*, 2615 – 2620.
[13] W. Schuhmann, T. J. Ohara, H. L. Schmidt, A. Heller, *J. Am. Chem. Soc.* **1991**, *113*, 1394 – 1397.
[14] S. Kunugi, Y. Murakami, K. Ikeda, N. Itoh, *Int. J. Biol. Macromol.* **1992**, *14*, 210 – 214.

[15] A. D. Ryabov, A. M. Trushkin, L. I. Baksheeva, R. K. Gorbatova, I. V. Kubrakova, V. V. Mozhaev, B. B. Gnedenko, A. V. Levashov, *Angew. Chem.* **1992**, *104*, 788–790; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 789–790.
[16] A. Badia, R. Carlini, A. Fernandez, F. Battaglini, S. R. Mikkelsen, A. M. English, *J. Am. Chem. Soc.* **1993**, *115*, 7053–7060.
[17] W.-C. Tsai, A. E. G. Cass, *Analyst* **1995**, *120*, 2249–2254.
[18] A. F. Bückmann, V. Wray, A. Stocker, *Methods Enzymol.* **1997**, 360–374.
[19] M. Tamura, T. Asakura, T. Yonetani, *Biochim. Biophys. Acta* **1972**, *268*, 292–304.
[20] A. D. Ryabov, V. N. Goral, *J. Biol. Inorg. Chem.* **1997**, *2*, 182–190.
[21] V. N. Goral, A. D. Ryabov, *Biochem. Mol. Biol. Int.* **1998**, *45*, 61–71.
[22] K. Schlögl, *Monatsh. Chem.* **1957**, *88*, 601–621.
[23] S. B. Brown, T. C. Dean, J. Jones, *Biochem. J.* **1970**, *117*, 733–739.
[24] E. B. Fleischer, J. M. Palmer, T. S. Srivastava, A. Chatterjee, *J. Am. Chem. Soc.* **1971**, *93*, 3162–3167.
[25] K. Kuzelová, M. Mrhalová, Z. Hrkal, *Biochim. Biophys. Acta* **1997**, *1336*, 497–501.
[26] F. W. J. Teale, *Biochim. Biophys. Acta* **1959**, *35*, 543.
[27] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **1951**, *193*, 265–275.
[28] P. George, *Biochem. J.* **1953**, *54*, 267–276.
[29] M. Gajhede, D. J. Schuller, A. Henriksen, A. T. Smith, T. L. Poulos, *Nature Struct. Biol.* **1997**, *4*, 1032–1038.
[30] F. Nastri, A. Lombardi, G. Morelli, G. D'Auria, C. Pedone, V. Pavone, *Chem. Eur. J.* **1997**, *3*, 340–349.
[31] A. J. Bard, L. R. Faulkner, *Electrochemical Methods. Fundamentals and Applications*, Wiley, New York, **1980**.
[32] C. Shi, F. C. Anson, *Inorg. Chem.* **1998**, *37*, 1037–1043.
[33] K. M. Kadish, J. Jordan, *J. Electrochem. Soc.* **1978**, *125*, 1250–1257.
[34] D. K. Das, C. Bhattaray, O. K. Medhi, *J. Chem. Soc. Dalton Trans.* **1997**, 4713–4717.
[35] T. Ruzgas, personal communication.
[36] N. C. Veitch, Y. Gao, A. Smith, C. G. White, *Biochemistry* **1997**, *36*, 14751–14761.
[37] R. E. Childs, W. G. Bardsley, *Biochem. J.* **1975**, *145*, 93–103.
[38] A. D. Ryabov, Y. N. Firsova, V. N. Goral, E. S. Ryabova, A. N. Shevelkova, L. L. Troitskaya, T. V. Demeschik, V. I. Sokolov, *Chem. Eur. J.* **1998**, *4*, 806–813.
[39] E. Monzani, L. Linati, L. Casella, L. De Gioia, M. Favretto, M. Gullotti, F. Chillemi, *Inorg. Chim. Acta* **1998**, *273*, 339–345.
[40] I. Hamachi, T. Matsugi, K. Wakigawa, S. Shinkai, *Inorg. Chem.* **1998**, *37*, 1592–1597.
[41] The latter value is probably set too high because of a very low Michaelis constant, which was not reliably determined in this case due to the experimental difficulties when operating with low concentrations of ferrocene derivatives.

Received: July 2, 1998 [F1237]