Novel Biocatalysts by Chemical Modification of Known Enzymes: Cross-Linked Microcrystals of the Semisynthetic Peroxidase Seleno-Subtilisin**

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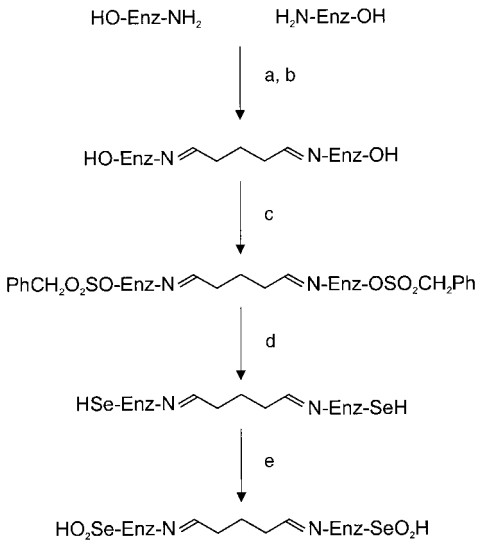
Dedicated to Professor Helmut Werner on the occasion of his 65th birthday

Although enzymes are widely accepted as valuable catalysts in synthetic chemistry their "natural" properties such as activity, selectivity, or stability are often insufficient. Several methodologies have been developed to overcome these problems. Genetic engineering techniques can improve the overall stability,[1] whereas new catalytically active sites are hard to achieve. Monoclonal catalytic antibodies opened many opportunities in this field, but it is difficult to obtain useful catalysts for asymmetric synthesis.[2] Finally, efforts were made to design synthetic enzyme mimics. However, the breakthrough in terms of catalytic efficiency and enantioselectivity still lies ahead.[2]

The oldest techniques for enzyme tailoring are "chemical" engineering methods. For instance, specific chemical modification of the catalytically active site yielded semisynthetic enzymes with completely changed catalytic properties.[3] Although semisynthetic enzymes open unique opportunities in enzyme design, this technique was mainly used as an analytical rather than as a synthetic tool. Here we report on a new type of chemically engineered semisynthetic enzyme for organic synthesis, which combines altered catalytic activity, reasonable substrate selectivity, and exceptional stability in one biocatalyst.

The serine proteinase subtilisin [3.4.21.62] is a well-known biocatalyst produced in several tons per year for industrial applications. Extensive substrate screenings, various genetically engineered mutants, and several X-ray studies have been described.[4] These detailed insights into the characteristics of subtilisin provided a sound basis for rational chemical engineering studies. In order to demonstrate the potential of chemical enzyme engineering, the active site of subtilisin as well as its peptide framework were modified by simple chemical reactions. We synthesized cross-linked crystals of the semisynthetic peroxidase seleno-subtilisin according to Scheme 1. Batch crystallization of subtilisin was achieved by slow addition of saturated sodium sulfate solution to a freshly prepared acetone precipitation and yielded microscopic needles (typically $100 \times 5 \times 5$ μm). Cross-linking of the crystals with glutardialdehyde effected that the material was insoluble in water as well as in organic solvents. Tüchsen and Ottesen[5] found that during a reaction time of 45 min three lysine residues at the subtilisin surface had reacted with its neighbors in the crystal lattice. X-ray studies showed the same

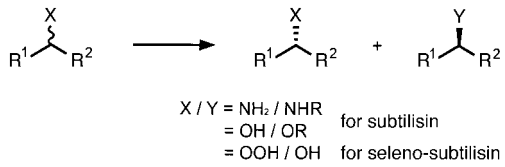


Scheme 1. Conversion of subtilisin into cross-linked microcrystals of seleno-subtilisin (see Experimental Section). a) Crystallization; b) glutardialdehyde; c) phenylmethanesulfonyl fluoride (PMSF); d) NaSeH; e) H_2O_2.

tertiary structure for cross-linked subtilisin and subtilisin in untreated crystals.[6]

Prior to seleno-modification the active site serine 221 had to be activated by phenylmethanesulfonyl fluoride (PMSF), a typical serine proteinase inhibitor. Nucleophilic substitution of the PMSF-substituted serine by sodium hydrogen selenide required two days treatment at 45 °C. Due to the insolubility of the enzyme preparation no chromatographic purification step could be applied during the synthesis. Hence, the enzyme crystals had to be washed with aqueous sodium borohydride solution to solubilize and remove the excess precipitated selenium. The selenol group at residue 221 was subsequently oxidized with hydrogen peroxide to yield the stable seleninic acid form of cross-linked crystals (CLC) of seleno-subtilisin.

As a result of the introduction of selenium into the protease subtilisin, the semisynthetic seleno-subtilisin revealed glutathione peroxidase activity,[7] and catalyzed the kinetic resolution of several racemic hydroperoxides[8, 9] (Scheme 2).



Scheme 2. Comparison of the catalytic activity of the protease subtilisin with that of the semisynthetic peroxidase seleno-subtilisin. Substrate- and stereoselectivity are comparable due to identical binding sites.[9, 14]

However, synthetic application of semisynthetic enzymes has to date been limited to 10-μmol scale and the chemically modified enzyme was not reusable. In contrast, CLC-seleno-subtilisin is insoluble in water and organic solvents and easily recovered after synthesis by centrifugation or filtration. Figure 1 summarizes the high level of activity and selectivity during ten reaction cycles. The kinetic resolution of racemic 2-hydroxy-1-phenylethyl hydroperoxide (**1**) was achieved in 25 – 30 min yielding 97 % *ee* on average. For instance, the

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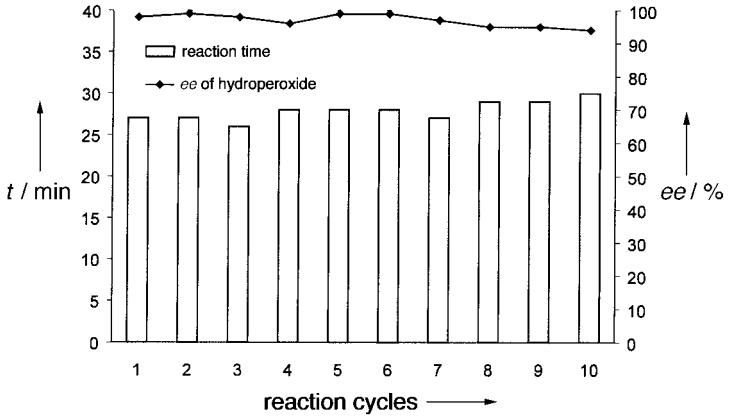

Figure 1. Enantioselective catalysis and recycling of CLC-seleno-subtilisin (see Experimental Section).

reaction of **1** or 1-phenylethyl hydroperoxide (**2**) was upscaled to 1-mmol scale without loss of activity or selectivity.

The exceptional characteristics of cross-linked enzyme crystals were discovered by Quiocho and Richards in 1964.[10a] They are stable towards varying pH values, high temperature, proteolytic degradation, and organic solvents, and kinetics of reactions are comparable to those of amorphous enzymes.[10] Accordingly, semisynthetic CLC-seleno-subtilisin revealed kinetic parameters ($k_{cat} = 458\ min^{-1}$, $K_m =$ 17.9 mM for **2**; assay see reference [8]) similar to those of amorphous peroxidase, and very high stability under denaturing conditions. Figure 2 shows a comparison of the activity of

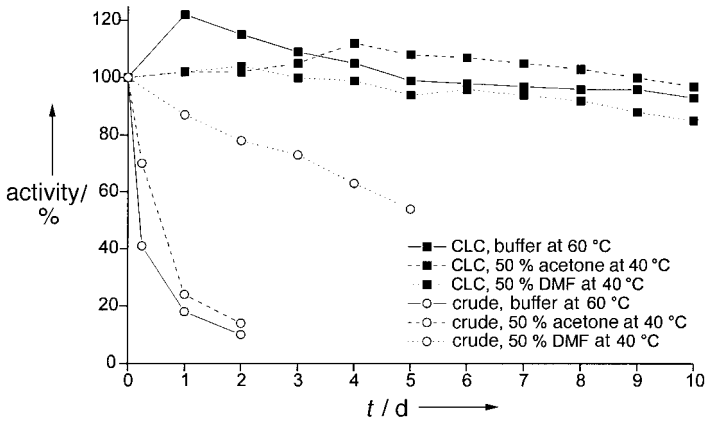

Figure 2. Stability of amorphous and cross-linked crystals of seleno-subtilisin in organic solvents or at high temperature. Peroxidase activity was measured photometrically at 410 nm with 0.2 mM cumene hydroperoxide and 0.2 mM TNB in 50 mM citric acid/NaOH buffer (pH 5.5) and 2 mM EDTA. The specific activities of crystalline (33 mU mg^{-1}) and amorphous (12 mU mg^{-1}) seleno-subtilisin were expressed as 100 %.

amorphous and CLC-seleno-subtilisin in different reaction media. In every case the cross-linked crystal lattice prevented rapid denaturation by unfolding mechanisms.

In summary, the cross-linked microcrystals of seleno-subtilisin are a new type of chemically engineered enzyme. For the first time we combined the methods of chemical modification of the enzymatic framework as well as the enzymatic active site. In comparison to other methods of enzyme development (e.g. genetic engineering or catalytic antibodies) only basic chemical equipment for the synthesis in gram-scale is necessary. Cross-linking of enzyme crystals yields an immobilized biocatalyst with exceptional stability. The high purity (crystalline enzyme!) together with the stability provide ideal prerequisites for further modification of the active site by chemical methods, resulting in new catalytic activity. By utilizing a well-known protein framework, details about substrate selectivity or affinity may be transferred and predicted from the template to the semi-synthetic enzyme.[9]

Experimental Section

Subtilisin Carlsberg (Novo Nordisk, 54 % (w/w) active protein) was prepurified by fractionated acetone precipitation and crystallized in 12 % Na_2SO_4 in 30 mM acetate/NaOH buffer (pH 5.5; 16 °C).[11] Treatment of subtilisin crystals with 1.5 % glutardialdhyde (45 min) in 30 mM KH_2PO_4/NaOH (pH 7.5) containing 13 % Na_2SO_4 resulted in cross-linked crystals (CLC) of subtilisin (25 % overall yield at gram-scale).[12]

Prior to the introduction of selenium, CLC-subtilisin was incubated with 5 mM PMSF for 1 h in 50 mM PIPES buffer (pH 7.0) containing 20 mM $CaCl_2$. Freshly prepared sodium hydrogen selenide[13] was added under argon atmosphere and the reaction was kept at 45 °C for 45 h. Precipitated red selenium was removed by washing three times with aqueous 25 mM $NaBH_4$ solution. The selenol form of CLC-seleno-subtilisin was oxidized to seleninic acid by treatment with 10 mM H_2O_2 (30 min at 20 °C). After repeated washing with 50 mM citric acid/NaOH buffer (pH 5.5) containing 20 mM $CaCl_2$, the enzyme crystals were stored in the same buffer and isolated for further experiments by centrifugation at 1000 g. Typically, 1.0 g CLC-subtilisin yielded 0.96 g CLC-seleno-subtilisin with 11 – 15 nmol active enzyme per milligram crystals (determined by active site titration with TNB[7]).

Enantioselective catalysis and recycling of CLC-seleno-subtilisin (see Figure 1). In 325 mL 30 mM citric acid/NaOH buffer (pH 5.5) and 2 mM EDTA, CLC-seleno-subtilisin (3.8 μmol) was preincubated with 2-nitro-5-thiobenzoic acid (TNB, 0.06 mmol; 15 min). 2-Hydroxy-1-phenylethyl hydroperoxide **1** (0.33 mmol) was added and the reaction was started by slow addition of TNB (0.33 mmol). CLC-seleno-subtilisin was recovered by centrifugation (1500 g). The hydroperoxide was extracted with diethyl ether and purified on a silica gel column. The *ee* of **1** was determined by HPLC on a Daicel-Chiralcel OB-H. Contrary to previous studies[8, 9] the blank reaction was negligible using CLC-seleno-subtilisin. Kinetic resolution of racemic hydroperoxide **1** or **2** was carried out accordingly in preparative 1-mmol scale yielding typically 42 % of the enantiomerically enriched hydroperoxides (for stereoanalytics and enantioselectivity cf.[8, 9]).

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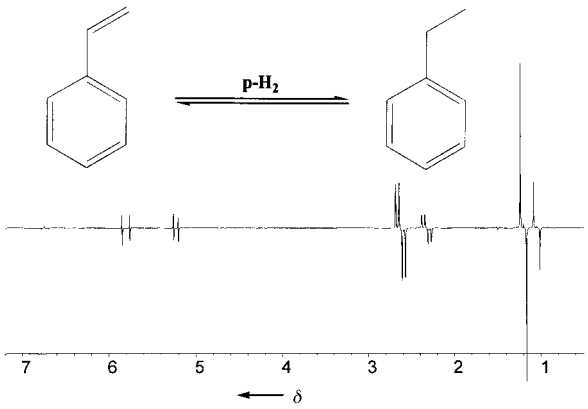

Figure 1. PHIP-NMR spectrum recorded 2 s after the end of the parahydrogen addition to styrene and $[Rh(cod)(dppb)]BF_4$ in $[D_6]$acetone.

Intermediate Product – Catalyst Complexes in the Homogeneous Hydrogenation of Styrene Derivatives with Parahydrogen and Cationic Rh^I Catalysts

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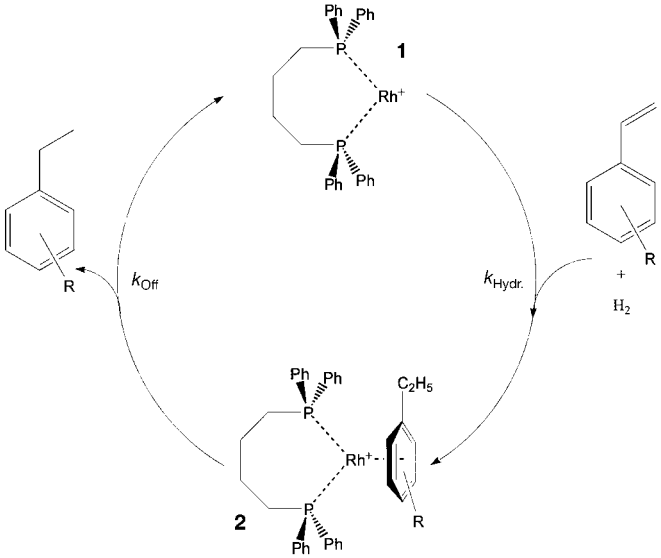
PHIP-NMR spectroscopy (PHIP = *p*ara*h*ydrogen *i*nduced *p*olarization) is an analytical method that provides profound insight into the mechanisms of homogeneously catalyzed hydrogenations, presupposed that the two parahydrogen nuclei remain *J*-coupled in the product molecule.[1–3] Recently, we have shown that PHIP-NMR spectroscopy is a suitable tool for the investigation of a pairwise hydrogen transfer into styrene, catalyzed by cationic Rh^I complexes.[4] In contrast to former assumptions,[5, 6] the corresponding catalytic cycle turned out to be partially reversible.[4] Herein, we report the PHIP-NMR spectroscopic detection of hydrogenation intermediates, which remain initially coordinated to the catalyst through their arene ring and then detach from the catalyst in a slow subsequent reaction step that influences the rate of the hydrogenation.

Addition of parahydrogen to a solution of styrene and the Rh^I precatalyst $[Rh(cod)(dppb)]BF_4$[7] in $[D_6]$acetone gave the PHIP-NMR spectrum shown in Figure 1. The product signals of ethylbenzene appear in the range of $\delta = 1-3$ (i.e., as an antiphase quartet at $\delta = 2.65$ and an antiphase triplet at $\delta = 1.20$).[8] In addition, high-field-shifted signals occur that show the same coupling constants as the signals of the free ethylbenzene. Evidently, they originate from product molecules just generated and still coordinated to the catalyst complex through their arene ring (Scheme 1). Their high signal intensities strongly suggest that the formation of the final hydrogenation product proceeds exclusively or predominantly via the intermediate product – catalyst complex.



Scheme 1. Simplified scheme for the hydrogenation turnover. The kinetic parameters k_{Off} and k_{Hydr} determine the degree of enrichment of the intermediate complex **2**. Saturation of free coordination sites at the Rh center with solvent or substrate molecules is not considered in this scheme.

A η^6 coordination of arenes to cationic Rh^I complexes has been extensively described before in the literature.[9a–f] For example, a slightly distorted η^6 coordination was found in the X-ray structure of $[Rh(P(OMe)_3)_2]BPh_4$.[9f] Likewise, the ^1H NMR spectrum of $[Rh(nbd)(C_6Me_6)]BF_4$[7] showed a singlet for the methyl protons over a wide range in temperature. The latter signal can, however, also be explained by a rapidly fluctuating η^4 coordination.[9a]

In the PHIP-NMR spectrum, the ratio of the catalyst attached to the free ethylbenzene (deduced from Figure 1) does not correspond to the thermodynamic equilibrium. If the delay between the end of the hydrogen addition and the detection pulse is extended to more than 30 s, this ratio

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