Artificial cofactors
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Chapter 3

Hydrogenation catalyzed by an artificial metalloenzyme based on penicillin acylase

Parts of the research described in this chapter was done by Dr. Lavinia Panella. The work was also done in collaboration with M. Jeronimus-Stratingh, J. Broos, A.J. Minnaard, J.G. de Vries, M.W. Fraaije and D.B. Janssen
Abstract

The possibility to generate an enantioselective catalyst by covalently incorporating achiral phosphite–rhodium complex into penicillin acylase of _E. coli_ was investigated. The semi-synthetic enzymes thus obtained were used to catalyze the hydrogenation of methyl 2-acetamidoacrylate. The hydrogenation proceeded to 100% conversion of the substrate but no enantiomeric excess was obtained.
Introduction

In recent years, biocatalysis has received increasing attention as an environmentally advantageous tool for the synthesis of enantiopure compounds (Faber, 2004). Although many intensive studies have been done in academia and industry, the number of applications of stereoselective enzymes in industry is still limited (Straathof et al., 2002). Recently, it has become possible to extend the application scope of biocatalysis by overcoming inherent limitations of enzymes, which are often related to their narrow substrate spectrum, low operational stability, restricted solvent tolerance, and limited enantioselectivity. High-throughput screening and directed evolution have become the most effective tools for the development of customized enantioselective enzymes (Reetz et al., 2006) and for improving enzyme stability (Lehmann and Wyss, 2001). Another issue that still restricts the range of possible applications is that enzymes are incapable of catalyzing many useful non-biological reactions, such as those catalyzed by transition metals (Trost, 1998). Transition metal catalysts play a crucial role in modern synthetic chemistry (Seebach, 1990). Enantioselective reactions catalyzed by transition metals have been extensively studied in the past four decades (Jacobsen et al., 1999). For example, rhodium (Rh) in combination with chiral phosphines is a frequently used catalyst for asymmetric hydrogenation, and the mechanism for Rh-catalyzed asymmetric hydrogenation of dehydroamino acids has been elucidated (Halpern, 1982). It has been shown that the coordination of the substrate to the metal center gives rise to a pair of diastereomeric catalyst-substrate adducts. The enantioselectivity is caused by the difference in the rate of addition of $\text{H}_2$ to the two diastereomers (Halpern, 1982).

The difficulty in design and synthesis of chiral ligands restricts the application of transition metal catalysts in asymmetric reactions. As a consequence, there is a limited number of efficient enantioselective metal catalysts that are used for industrial applications (Blaser et al., 2001; 2003). In addition to the use of chiral metal complexes, a possible strategy to design selective catalysts is to incorporate nonspecific achiral catalytic moieties into the chiral environment of a protein cavity. The advantage of this strategy is the avoidance of the cumbersome and lengthy synthesis of the chiral ligands. The first hybrid biocatalysts were constructed by chemically modifying a protein by covalent or non-covalent anchoring of a metal–ligand moiety (Kaiser & Lawrence, 1978; Wilson & Whitesides, 1978). Inspired by the milestone work with flavopapain, several research groups have recently developed hybrid catalysts with promising properties by covalently incorporating transition-metal catalysts to proteins (Lin, 1999; Qi, 2001; Reetz, 2002; Nicholas, 2002; Davis, 2003; Skander, 2004). However, when papain was used as the host protein for an achiral rhodium-phosphite complex (Fig. 1) to catalyze the hydrogenation of methyl 2-acetamidoaerylactate, no enantiomeric excess was obtained (Panella et al., 2005; 2006). The cavity of papain was thought to be so wide that the covalently incorporated transition-metal catalyst was very flexible and surrounded by solvent molecules. Therefore, besides the covalent linkage, there are probably insufficient extra contacts between the catalyst and the protein that would provide a chiral environment for stereodiscrimination.
In order to test the concept of artificial metalloenzymes further, other protein candidates with narrow cavities should be selected. In this study, penicillin acylase of *E. coli* (E.C 3.5.1.11) (PA) is chosen as the protein host for the transition-metal catalyst. PA catalyzes the hydrolysis of benzylpenicillin to give 6-aminopenicillanic acid, an intermediate in the production of semi-synthetic penicillin (Cole, 1969). The gene that encodes PA was cloned in 1986 (Schumacher et al., 1986). The enzyme is produced as a pre-pro-protein, consisting of a 26 amino acid leader peptide, a 201 amino acid α-subunit, a 62 amino acid connecting spacer peptide, and a β-subunit of 557 amino acids. With the aid of the leader peptide, the pre-pro-protein is directed to the periplasmic space. The leader peptide is cleaved off during translocation over the cell membrane. The removal of the spacer peptide in the maturation process yields the active form of PA. The active form is a kidney-shaped heterodimer consisting of a 23 kDa α-subunit and a 63 kDa β-subunit (Duggleby et al., 1995; Brannigan et al., 1995).

The substrate binding site is located in a crevice formed by an αβα core structure (Duggleby et al., 1995). The X-ray structure of the complex of PA with p-nitrophenol shows that the side chains of several hydrophobic residues from both the α- and the β-subunit form a narrow pocket which functions as the binding site for the acyl moiety of the substrate (Fig. 2). βSer1 is critical for the processing of the mature enzyme and for the activity of PA (Duggleby et al., 1995). This N-terminal nucleophilic serine is positioned at the end of an anti parallel β-sheet of the αβα core. βSer1 can be modified with PMSF and thereby the enzymatic activity is inhibited (Kabakov et al., 1995). This indicates that the transition-metal catalyst could be covalently incorporated into the catalytic residue of PA by reaction with βSer1. A PA mutant, in which αMet142 in the acyl binding site is mutated to a cysteine (αM142C), was constructed by Drs. S. Jager in an earlier study. The free cysteine can also be modified with a reactive group bearing a catalyst.

![Fig. 1. The structure of the phosphite ligand complexed with Rh(I).](image1)

![Fig. 2. The structure of wild-type PA with p-nitrophenol in the active site. Left: surface of the active site of wild-type PA with p-nitrophenol (black) bound. Right:](image2)
In this research, both wild-type PA and the αM142C PA mutant were selected for the modification with the phosphite ligand. It was assumed that the cavity of PA might induce enantioselectivity in three different ways: 1) by providing an extra coordination for the unsaturated Rh-monophosphite complex; 2) by forcing the structurally flexible phosphite backbone in a preferred conformation; 3) by favoring a specific complexation of the substrate to the catalyst due to the protein environment (Panella, 2006). We report on the use of wild-type PA and the αM142C PA mutant as host proteins for a rhodium-phosphite complex. The resulting artificial metalloenzymes are tested in the enantioselective hydrogenation of methyl 2-acetamidoacrylate.

**Experimental procedures**

**Chemicals**

Papain from papaya latex was obtained from Sigma-Aldrich as a buffered suspension in 50 mM sodium acetate (NaAc), pH 4.5, containing 0.01% thymol. Iodoacetamide was purchased from Sigma-Aldrich. Sequencing grade modified trypsin was obtained from Promega. Dithiothreitol (DTT) was obtained from Roche, phenacyl bromide (phenacyl bromide) was purchased from Fluka and 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB) was obtained from Drs. S. Jager.

**Expression and purification of PA**

Cells of *E. coli* HB101 harboring the cloned *Escherichia coli* wild-type PA gene (accession number 42247) or the αM142C mutant gene were grown in LB medium at 17 °C and rotary shaking at 150 rpm. Cells were harvested in the late exponential phase by centrifugation at 5,000 g for 15 min. Periplasmic extract was prepared as follows. The cells were resuspended in 1/10 (100 mL) of the original culture volume (1 L) of ice-cold osmotic shock buffer (20% sucrose, 100 mM Tris-Cl, 10 mM EDTA, pH 8.0) and centrifuged at 5,000 g for 15 min. Subsequently, the cells were resuspended in 1/10 volume of the original culture volume of ice-cold 1 mM EDTA and again centrifuged at 5,000 g for 15 min. Potassium phosphate buffer (1 M, pH 7.0) was added to the supernatant to a final concentration of 50 mM which yielded a buffered periplasmic extract.

For further purification of PA, (NH₄)₂SO₄ was added to the periplasmic extract to a final concentration of 1.5 M. The sample was loaded on a Resource Phe column (GE Healthcare Life Sciences) and eluted with a linear gradient of 1.5 M to 0 M (NH₄)₂SO₄ in 20 mM potassium phosphate buffer of pH 7.0. PA eluted at a concentration of 1 M (NH₄)₂SO₄. Fractions containing the active enzyme were pooled, and by using Amicon ultrafiltration cell with a YM 30 filter (Millipore, USA) the buffer system was replaced with 50 mM potassium phosphate, pH 7.0, containing 5% glycerol. The concentration of purified enzyme was determined by measuring the A₂₈₀ using a molar extinction coefficient of 210,000 M⁻¹ cm⁻¹ (Alkema et al., 1999). The yield was 1.7 mg purified PA per 1 culture volume.
For lyophilization of PA, a PA solution (1.0 mg/mL) in Eppendorf tubes was frozen in liquid nitrogen and placed in a lyophilizer tube and subjected to vacuum environment of 0.05 mbar for 24 h.

Expression and purification of flavodoxin
Cells of *E. coli* TG2 harboring the cloned *Azotobacter vinelandii* flavodoxin II gene were grown at 37 °C and rotary shaking at 150 rpm. Cells from 1 L culture were harvested by centrifugation at 4,000 g (Beckman J2-21 ME centrifuge with a JA-10 rotor), and resuspended in 25 mL potassium phosphate buffer, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM MgSO₄ (pH 7.0). Cells were disrupted by sonication at 20 KHz for 20 min at 4°C. Following centrifugation at 23,000 g (Beckman J2-21 ME centrifuge with a JA-17 rotor) to remove cellular debris, the supernatant was applied onto a Q-Sepharose column pre-equilibrated in the same buffer. The enzyme was eluted with a linear gradient from 0 to 1.0 M KCl in the same buffer. Yellow fractions were pooled, desalted and concentrated in an Amicon ultrafiltration unit (Millipore, Billerica, MA, USA) equipped with a YM-30 membrane.

Modeling the ligand into the PA active site
Manual docking of the ligand into the PA active site was done using PyMol (DeLano, 2002). A structure for the ligand (smiles COCCOCOCOCoc4cc(c(C)(C)(C)ccc)3op(Oc1cccc(C(=O)c2cc(C(C)(C)(C)ccc(O COCCOCOCOC)cc2c3c4)) was generated using the Online Smiles Translator and Structure File Generator at http://cactus.nci.nih.gov/services/translate/. The Br atom of the ligand was superimposed on the OSer1 OG of PA and rotatable bonds were edited manually in the PyMol editing mode to obtain a suitable fit according to visual inspection.

Automated ligand docking simulations were performed using AutoDock 4.0 (Goodsell and Morris, 1990; Morris *et al*., 1990) running under Cygwin/Windows XP on a 4Ucom PC. Ligand and protein files were created using AutoDockTools. The ligand structure used was that of the rhodium-free ligand with the CH₂Br group replaced by a -NH₂ group. Docking runs were performed with all 26 possible ligand bonds set flexible and the CA-CB-OG bonds of βSer1 of PA also set flexible. A grid box was defined that encompasses the substrate binding cave of PA and 50 runs were done using the Lamarckian genetic algorithm. The best fits were inspected using AutoDockTools and visualized using PyMol.

Activity assay of PA
Enzymatic activity was determined by measuring the initial velocity of hydrolysis at a saturating concentration (250 μM) of 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB) in 50 mM potassium phosphate buffer (pH 7.0) at 25 °C. The release of 3-amino-6-nitrobenzoic acid (NABA) was followed by measuring the increase in the absorbance at 405 nm in a Lambda Bio40 UV/VIS spectrometer (Perkin Elmer). Rates were calculated using a Δε of 9.09 mM⁻¹ cm⁻¹ (Alkema *et al*., 1999).

Thiol titration of aM142C PA
The thiol group of aM142C PA was estimated by titration with 5-(octyldithio)2-nitrobenzoate as described (Faulstich *et al*., 1993), both before and after inactivation of the enzyme by phenacyl bromide. Reactions were performed by measuring the
release of 5-thio-2-nitrobenzoate anion (ε₄₁₂ = 13,600 M⁻¹cm⁻¹) in a Lambda Bio40 UV/VIS spectrophotometer (Perkin Elmer). The free thiol of active αM142C PA (10 μM) was titrated by adding 5-(octyldithio)-2-nitrobenzoate (1.0 mM) in 50 mM phosphate buffer, pH 6.2, 1 mM EDTA, and 0.1 M NaCl. After incubation of the active enzyme (10 μM) with phenacyl bromide (2 mM) for 30 min, the protein was purified by gel filtration and concentrated. The free thiol of this modified αM142C PA (10 μM) was titrated in the same way.

Modification of wild-type PA and αM142C PA with phenacyl bromide in phosphate buffer
Enzyme (10 μM) was incubated with phenacyl bromide (phenacyl bromide) (2 mM) in 20 mM potassium phosphate buffer containing 10% methanol, pH 7.0, at 25 ºC. At various times, aliquots of 10 μL were drawn from the reaction mixture and assayed for enzymatic activity using 250 μM NIPAB as the substrate.

Modification of wild-type PA, αM142C PA, papain and flavodoxin with the phosphite ligand in phosphate buffer containing cosolvents
The phosphite ligand (Fig. 1) was prepared by L. Panella (Panella et al., 2005). Before modification of the proteins, a stock solution of the ligand (10 mg/mL) was prepared by dissolving it in DMSO or other another cosolvent used for the modification of PA. Enzyme (10 μM) was incubated with the phosphite ligand (0.2-0.4 mM) in potassium phosphate buffer (20 mM, pH 7.0) containing different cosolvents for 24-48 h. At various times aliquots were drawn from the reaction mixture for activity assays.

Extraction of PA into isooctane with ionic surfactant
For the solubilization of PA in isooctane, PA was extracted into the organic phase as described (Paradkar and Dordick, 1994 and Wangikar et al., 1997). The enzyme was extracted from an aqueous solution (pH from 5.0 to 8.8) with an equal volume of isooctane containing 20, 50, 100, or 200 mM aerosol-OT (AOT). The aqueous phase contained wild-type PA (0.1-0.2 mg/mL), and either sodium acetate buffer (50 mM, pH 5.0), sodium phosphate buffer (10 mM, pH 6.3 or pH 7.0), or Bis-tris propane (1,3-bis(tris(hydroxymethyl)methylamino)propane) buffer (10 mM, pH 7.8 or 8.8), and various concentrations of NaCl. The two solutions were mixed by stirring at 25°C for 2 min and centrifuged to obtain clean phase separation. For the determination of the content of wild-type PA in isooctane, 10 μL of ionic surfactant modified enzyme in isooctane was added to 1.0 mL phosphate buffer (50 mM, pH 7.0) and the absorbance at 280 nm and activity against NIPAB were recorded.

Modification of lyophilized wild-type PA with the phosphite ligand in organic solvents
For the test of the modification of wild-type PA in different solvents, the lyophilized enzyme (1.0 mg) was suspended in 1 mL organic solvent. The phosphite ligand was dissolved in acetone and added to the wild-type PA suspension to 1.2 mg/mL. The suspension was shaken at 200 rpm at 25 °C or 37 °C for 24 to 48 h. The suspended protein was pelleted in a centrifuge. After removal of the solvent, the protein was washed three times with n-propanol and dissolved in 0.5 mL 100 mM MOPS, pH 7.0.

For the hydrogenation reactions, 7-13 mg wild-type PA was suspended in 1.0 mL acetone containing 6.1-6.6 mg ligand and incubated at 37°C, 200 rpm, for 20 h.
After modification with the ligand, the enzyme was pelleted in a centrifuge and washed with n-propanol for three times and then dissolved in MOPS buffer (100 mM, pH 7.0) for further complexation with rhodium(I). The concentration of modified wild-type PA was determined using coomassie brilliant blue.

**Complexation of the cofactor-modified wild-type PA and Rh\((\text{COD})_2\text{BF}_4\)**

Ultrafiltration of the modified wild-type PA solution was carried out in an Amicon YM-10 filter (Millipore) to replace the MOPS buffer with H\(_2\)O. Then, bis(1,5-cyclooctadiene)rhodium(I) tetrafluoroborate (Rh\((\text{COD})_2\text{BF}_4\)) (8 mM in 1,4-dioxane) was added to the modified wild-type PA solution at various Rh/PA ratios. The solution was gently stirred at room temperature for 1 h and then loaded onto an Econo Pac 10 DG disposable chromatography column (Bio-Rad) and washed with potassium phosphate buffer (25 mM, pH 7.0) to remove the unbound Rh\((\text{COD})_2\text{BF}_4\).

**Hydrogenation reactions catalyzed by PA-Phos-Rh**

Hydrogenation reactions were done by L. Panella. For the hydrogenation reactions, a glass vial was filled with 2 mL of degassed buffer solution (phosphate buffer, 25 mM, pH 7.0) (homogeneous catalysis) or n-propanol (heterogeneous catalysis) containing PA-Phos-Rh (50.0 μM) and methyl 2-acetamidoacrylate (40 mM). The glass vial was put in an autoclave and after flushing with N\(_2\) the system was pressurized with hydrogen (12 bar) and the reaction mixture was stirred at room temperature for 16 h. The reaction was stopped by release of the H\(_2\) pressure. The resulting mixture was extracted with ethylacetate (3×5 mL) and the combined organic layers were dried with Na\(_2\)SO\(_4\). Conversion was determined by \(^{1}\)H NMR and GC. Enantiomeric excess was analyzed by capillary chiral GC (CP Chirasil-L-Val column, 25 m × 0.25 mm × 0.25 μm).

**In-gel tryptic digestion of modified PA**

The modified PA was separated by electrophoresis in a 12% SDS polyacrylamide gel and stained with coomassie brilliant blue. The selected protein bands in the gel were cut with a clean scalpel and placed in an Eppendorf tube. The gel pieces were first washed with 400 μL H\(_2\)O for 1 h, and then washed several times with 300 μL 70% 25 mM NH\(_4\)HCO\(_3\) containing 30% acetonitrile until the gel pieces were colorless. The gel pieces were further washed with 300 μL 50% 25 mM NH\(_4\)HCO\(_3\) containing 50% acetonitrile for 30 min and dried in a SpeedVac vacuum centrifuge. For the digestion, 20 μL of sequencing grade trypsin (Promega, US) (10 ng/μL in resuspension buffer, provided with the trypsin) was added to the gel pieces and the mixture was incubated at 37 °C overnight. For peptide extraction, the trypsin solution was removed and the peptides were extracted from the gel pieces with 10 μL of 75% acetonitrile containing 1.25% formic acid.

**ESI-MS of the modified PA, papain and flavodoxin**

Electrospray mass spectrometry (ESI-MS) was performed on an API3000 a triple quadrupole mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada), equipped with an atmospheric pressure ionization source and a TurbolonSpray interface. The samples were diluted with an aqueous solution of methanol (85%) containing 0.01% formic acid. The spectra were scanned in the range between m/z 400 and 1600.
Hydrogenation catalyzed by an artificial metalloenzyme based on penicillin acylase

Results

Modification of PA with the linker in aqueous buffer

Our previous results with modification of papain with the phosphate ligand (Fig. 1) indicated that the active site of the enzyme is too spacious for providing a sterically-restrained binding site that could introduce selectivity. Since the active site of PA is a deep cave from the protein’s surface, we selected this enzyme as an alternative. PA has a reactive serine as the nucleophile instead of a cysteine, which also circumvents the problem of thiol oxidation that can complicate the use of papain. Using manual docking it was found that sufficient space exists in the active site for positioning the ligand in such a way that the bromine atom superimposes with the OG of βSer1, indicating that a coupling reaction may occur (Fig. 3). Computer docking of the ligand was attempted with Autodock 4.0, using a model ligand in which the CH₂Br group of the ligand was replaced by an –NH₂ group. The lowest energy pose of 50 runs positioned the ligand deep into the active site cave with the coupling site positioned close to the reactive oxygen of βSer1 (Fig. 3). Furthermore, the docking pose observed in Fig. 3 suggested that there may be space for rhodium to bind to the phosphite group after the ligand is bound to the enzyme.
Fig. 3. Modeling of ligand binding into the active site of PA. A: figure generated by manual docking using PyMol, showing that there is sufficient space for the ligand to bind to βSer1. No obvious steric overlap between the ligand and the walls of the active site cave is observed. There is also open space left between the enzyme surface and the phosphorus involved in rhodium binding. B: modeling of a model ligand (see Materials and Methods, stick representation) binding into the active site using...
AutoDock 4.0. The best docking pose for coupling of the model ligand is shown. Here the open space for rhodium binding is reduced. C: a side view of another good AutoDock pose shows the proximity (distance 2.8 Å) of the -NH$_2$ of the model ligand and the $\beta$Ser1 OG of the enzyme. The ligand and flexible Ser sidechain are shown in stick representation. The difference between A and B shows that there is large conformational freedom for the ligand in the PA active site.

Before the modification of wild-type PA with the phosphite ligand, the possibility to modify this protein was first tested with phenacyl bromide, which mimics the linker part of the ligand. For this, purified enzyme was incubated with phenacyl bromide at room temperature. As shown in Fig. 4A, the activity was decreased to 32 % after 6 h. When incubation was conducted at 4 °C, the activity further decreased, leaving 3.7% of the initial activity after 48 h at 4 °C. This inhibition process is much slower than the modification of papain, which took only 4 h to reach complete inhibition of activity at room temperature (Panella et al., 2005).

![Fig. 4. The inhibition of PA by phenacyl bromide. Panel A: inhibition of wild-type PA (10 $\mu$M) with phenacyl bromide (2 mM) in potassium phosphate (20 mM, pH 7.0) containing 10% DMSO. Panel B: inhibition of $\alpha$M142C PA (10 $\mu$M) with phenacyl bromide (2 mM) in potassium phosphate (20 mM, pH 7.0) containing 10% DMSO. Symbols: (▲) enzyme incubated with phenacyl bromide; (●) enzyme without phenacyl bromide.](image)

A cysteine mutant of PA ($\alpha$M142C PA) was also tested with phenacyl bromide. Methionine 142 in the $\alpha$ chain of PA is in the binding site for the acyl moiety of the substrate. The binding site is a narrow pocket formed by the side chains of several hydrophobic residues (Duggleby et al., 1995). The modification of M142 may block substrate binding and thus inhibit the enzymatic activity. Mutant $\alpha$M142C PA was expressed, purified and modified in the same way as with wild-type PA. The activity of the unmodified $\alpha$M142C-PA was about 40% of that of the wild-type enzyme. The modification of $\alpha$M142C-PA was followed by checking the enzyme activity during the incubation of the enzyme with the linker and comparing the number of free thiols before and after the inactivation. Fig. 4B shows the decrease of the activity of $\alpha$M142C-PA (10 $\mu$M) during the process of modification with phenacyl bromide (2}
mM) at room temperature. The activity of αM142C-PA dropped to 9% and no thiol was detected 30 min after the addition of phenacyl bromide. After 3 h, the activity of αM142C-PA decreased to 2.8% and again no free thiols were detected. The faster modification of αM142C-PA as compared to wild-type PA may be due to the fact that the cysteine lies in the hydrophobic acyl binding site which has a higher affinity for the phenacyl group than the active site βSer1 (Fig. 2). The covalent coupling of the phenacyl group to Cys142 will prevent binding of substrate in the active site and thus inhibit the activity. However, the possibility of modification of βSer1 could not be excluded and thus mass spectrometry was used to determine the position of the modification.

In order to confirm the modification of PA by mass spectrometry, samples of PA modified with the linker and the phosphite ligand were analyzed by MS. Since PA is a heterodimer, the mass of each subunit cannot be directly determined by ESI-MS. Because the separation of the two subunits by gel filtration under denaturing conditions failed, we could not determine the mass of the β-subunit and the modification. Then, we turned to the determination of the mass of the peptide which contained the amino acid residue that was expected to be modified by the linker or the phosphite ligand. The LC-MS spectrum of the trypsin-digested wild-type PA treated with phenacyl bromide showed that the enzyme was indeed covalently modified (Fig. 5). A singly charged ion with \( m/z \) of 935.0 corresponding to the peptide SNMWVIGK, which contains βSer1, was found in the mass spectrum. After modification with phenacyl bromide, an ion with \( m/z \) of 1053.0 corresponding to the same peptide plus acetophenone (\( C_8H_7O, 118 \) Da) was found, indicating that wild-type PA was modified. When the modification was carried out with the complete ligand instead of the linker only, the SNMWVIGK peptide that was expected to undergo modification was no longer visible in the mass spectra (data not shown), although other peptide fragments were present in the normal way, which confirms that the peptide was modified and suggests that the modification interfered with the mass spectrometric analysis.
Fig. 5. ES/MS-spectra of the tryptic peptide containing the βSer1 generated from PA after labeling with phenacyl bromide and without labeling. The MS spectra of the peptide βSer1-βLys8 are shown. The peptides were obtained from unlabeled enzyme (panel A) or from PA preincubated with phenacyl bromide (panel B). Cps: counts per second; amu: atomic mass unit.

For αM142C PA, the ions that correspond to peptide SNMWVIGK (935.0 Da), which contains βSer1, and peptide WEPFDVAMIFVGTCANR (2013.0 Da), which contains the introduced cysteine, were found but neither of the peptides carrying the modification with phenacyl bromide could be found in the spectrum. Although no mass spectrometric proof was obtained for the modification of αM142C PA, it was still decided to continue the modification with the phosphite ligand both with the wild-type PA and the mutant enzyme.

Modification of PA with the phosphite ligand in aqueous buffer containing cosolvents

Because the phosphite ligand was not solubilized very well in the phosphate buffer, even though the ethylene glycol groups are designed to increase solubility, cosolvents were added to improve the solubility of the ligand in aqueous solutions. For this, different cosolvents (DMSO, dioxane, methanol, glycerol, acetone, ethylene glycol, 1,2-dipropandiol) at various concentrations were tested. However, the addition of cosolvent improved the solubilization of the ligand in aqueous solutions only slightly, which means that a high concentration of cosolvents is needed to fully solubilize the ligand in water. For example, the turbid solution of 0.2 mM ligand in potassium phosphate buffer (20 mM, pH 7.0) became clear only when 4 volumes of the cosolvent (DMSO or glycerol) were added and the ligand concentration was diluted to 40 μM accordingly.

For most cosolvents, the solubility of the ligand was improved with increasing the cosolvent concentration, as judged by visual inspection. However, in several cases the stability of the enzyme decreased as shown by activity measurements. The enzyme was stable in 80% glycerol, 30% 1,2-propanediol, 10% DMSO and 40% acetone with a loss of less than 20% of the initial activity during 6 h incubation at room temperature. Increase of the cosolvent concentration inactivated PA rapidly. For example, when lyophilized PA was dissolved in 100% glycerol, it showed a half-life of 4 h. The protective effect of alcohols is correlated with the number of hydroxyl groups (Azevedo et al., 1999). The addition of monohydric alcohols usually causes a stronger decrease in the stability of a native protein structure than diols and triols. This decrease is caused by interactions of these alcohols with hydrophobic moieties which are more accessible in the denatured state than in the native state of proteins (De Cordt et al., 1994). In this case, glycerol seems to be a good solvent for the modification because the stability of the enzyme and the solubility of the ligand are best compromised. But the high viscosity of glycerol might also limit the mass transfer.

When wild-type PA and αM142C PA (10 μM) were incubated with 40-fold excess of ligand (0.4 mM) in potassium phosphate buffer containing 30% 1,2-propanediol or 10% DMSO (Fig. 6), the solution was turbid and no inhibition of enzyme activity was observed within 16 h. The turbidity may be due to incomplete dissolution or surface active properties in solvent mixtures. When the modification was attempted with the ligand in 80% glycerol, the solution was slightly turbid and no
inhibition of wild-type PA or \( \omega \text{M142C PA} \) by the ligand was observed after 16 h. In
40% acetone, the enzyme activity decreased to 20% after 16 h incubation with the
ligand while the untreated enzyme still kept 80% of its activity in 40% acetone (Fig.
7). However, this only happened when a very small reaction volume (25 \( \mu \text{L} \)) was used.
When the reaction was scaled up to 1.0 mL, no inhibition of PA activity was observed
for unknown reasons.

The failure of the modification of PA with the phosphite ligand in aqueous
solutions could in principle be caused by the degradation of phosphite ligand. In order
to check the quality of the phosphite ligand used for the modification reactions with
PA, papain and flavodoxin which have accessible cysteine residues, were treated with
the phosphite ligand from the same batch. SDS-PAGE and MS analysis showed that
both proteins were modified. In a denaturing gel, flavodoxin modified with the
phosphite ligand showed two close bands indicating that part of the protein was
covaletely modified (Fig. 8). ESI-MS data showed an increase of 785 Da of both
papain and flavodoxin which corresponds to the mass of the phosphite ligand (Table
1). These results showed that the phosphite ligand was still reactive. The difficulty in
the modification of PA with the phosphite ligand can thus be attributed to the fact that
the active site of PA is not open enough for the phosphite ligand to enter and react,
especially because of the two bulky isobutyl groups of the ligand.

Fig. 6. Effect of inhibition of wild-type PA(10 \( \mu \text{M} \)) with the phosphite ligand (2 mM)
in potassium phosphate (20 mM, pH 7.0) containing 10 % DMSO. Symbols: (\( \Delta \))
enzyme incubated with the phosphite ligand; (\( \bullet \)) enzyme without the phosphite ligand.
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Fig. 7. The inhibition of wild-type PA (10 \( \mu \)M) with the phosphite ligand (2 mM) in Tris-HCl (50 mM, pH 8.0) 40% acetone. Symbols: (▲) enzyme incubated with the phosphite ligand; (■) enzyme without the phosphite ligand.

Fig 8. SDS-PAGE of native flavodoxin (lane 4, 8), flavodoxin modified with the phosphite ligand (lane 5-6), flavodoxin modified with the liker (lane 7) and the low molecular weight protein marker (lane 1).
Table 1. Results of the ESI-MS analysis. Masses (Da) of observed and expected peptides are shown.

<table>
<thead>
<tr>
<th>Protein</th>
<th>expected (Da)</th>
<th>observed (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain</td>
<td>23,422</td>
<td>23,454</td>
</tr>
<tr>
<td>Papain modified with the phosphite ligand</td>
<td>24,207</td>
<td>24,207</td>
</tr>
<tr>
<td>Flavodoxin (apo flavodoxin+ FMN)</td>
<td>20,107</td>
<td>20,110</td>
</tr>
<tr>
<td>Flavodoxin modified with the phosphite ligand</td>
<td>20,892</td>
<td>20,895</td>
</tr>
</tbody>
</table>

Partial unfolding of the protein might help the covalent attachment of a cofactor in a cavity because the modification site becomes more accessible (Kuang et al., 1996; 1997). To try this, wild-type PA and αM142C PA were incubated in 1-3 M urea to become partially unfolded. Both the wild-type and the mutant enzyme could withstand urea at this concentration without significant loss of activity (96% residual activity after 4 h at 25°C in 3 M urea). However, again no inhibition of activity caused by the phosphite ligand was observed when wild-type PA and αM142C PA were incubated with the cofactor in 1-3 M urea and either 50% glycerol or 10% DMSO. The reason might be that PA can resist these concentrations of urea without increasing the flexibility of the residues that form the active site cavity and without significant opening of the active site.

**Extraction of PA into isooctane with the ionic surfactant**

Because the modification of wild-type PA and αM142C PA with the phosphite ligand was difficult, it was considered that it could be helpful to increase the cofactor concentration. In order to circumvent the solubility problem of the ligand in aqueous buffer, the modification of PA was performed in organic solvents. One of the methods that can be used is the solubilization of proteins in an organic solvent in the presence of surfactants that form reversed micelles or ion paired protein-surfactant complexes (Wolbert et al., 1989; Matzke et al., 1992). In a study on the catalytic activity of PA, the subunits of PA were solubilized in isooctane by reversed micelles of the surfactant aerosol OT (di-2-ethylhexyl sodium sulfosuccinate, AOT) and were found to have catalytic activity (Kabakov et al., 1991, 1992, 1994, 1995; Alves et al., 1995). Another study on PA in a reversed micelle system of AOT-H₂O-octane showed that the enzyme loses no more than 20% of its initial activity during 3-4 h in the reversed-micelle systems and retains its catalytically active structure (Shamolina et al., 1999).

The structure of AOT is shown in Fig. 9. The charged head groups of AOT molecules bind to the surface of the protein by electrostatic attraction, while the hydrophobic lipid tail group of AOT partitions from the aqueous-organic interface into the bulk organic phase. Depending on the protein:AOT ratio, the protein can reside either in a reversed micelle or form a hydrophobic ion-paired complex with AOT (Paradkar and Dordick, 1994). The reversed micelle is favored over the hydrophobic ion-paired protein-AOT complex when excess AOT is present. If AOT is not in excess, the ion-paired form is favored. Because electrostatic interactions play an important role in the extraction of protein with ionic surfactants into organic solvents, the major parameters that influence the extraction are pH, ionic strength and
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structural properties of the protein (molecular weight, isoelectric point, overall charge and charge distribution) (Wolbert et al., 1989).

Fig. 9. Structure of aerosol OT.

Various conditions were tested to extract wild-type PA into isooctane (Table 2). In 10 mM phosphate buffer (pH 6.3) containing 50 mM NaCl, 28% (0.28 mg out of 1.0 mg) of wild-type PA was extracted to the organic phase containing 100 mM AOT. At such a high concentration of AOT (100 mM), PA is predicted to reside in reversed micelles formed by AOT. However, because the concentration of AOT in isooctane was not determined in the water phase and isooctane phase due to the difficulty in the development of an assay for AOT concentrations, the formation of reversed micelles could not be confirmed. PA solubilized in isooctane was stable at room temperature with only 10% loss of activity in 24 h, allowing a long incubation time with the ligand in isooctane. There was no solubility problem of the ligand in isooctane. However, when PA entrapped in AOT was incubated with the phosphite ligand (10 mM), no inhibition of enzyme activity by the ligand was observed after 20 h at room temperature. The reason might be that the isobutyl group of the ligand was too bulky to enter the active site of PA.

Table 2. PA extraction to isooctane with AOT.

<table>
<thead>
<tr>
<th>pH (mM)</th>
<th>AOT (mM)</th>
<th>20 mM</th>
<th>50 mM</th>
<th>100 mM</th>
<th>200 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3</td>
<td>20</td>
<td>3.5</td>
<td>2.1</td>
<td>10.3</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>isooctane phase</td>
<td>96.5</td>
<td>97.9</td>
<td>89.7</td>
<td>88.0</td>
</tr>
<tr>
<td></td>
<td>water phase</td>
<td>97.6</td>
<td>81.7</td>
<td>71.8</td>
<td>83.6</td>
</tr>
<tr>
<td>50</td>
<td>isooctane phase</td>
<td>2.4</td>
<td>18.3</td>
<td>28.2</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>water phase</td>
<td>97.8</td>
<td>90.2</td>
<td>86.5</td>
<td>85.6</td>
</tr>
<tr>
<td>100</td>
<td>isooctane phase</td>
<td>2.2</td>
<td>9.8</td>
<td>13.5</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>water phase</td>
<td>97.8</td>
<td>90.2</td>
<td>88.8</td>
<td>94.0</td>
</tr>
</tbody>
</table>

The concentration of PA in aqueous buffer was 1.0 mg/mL. For measurement of PA activity in the organic phase, 10 μL of enzyme modified with ionic surfactant in isooctane was added to 1.0 mL phosphate buffer (50 mM, pH 7.0) containing NIPAB (250 μM), and the activity of PA was measured at 405 nm using NIPAB as the substrate. For measurement of PA activity in the water phase, 10 μL of solution was taken from the water phase after the extraction of PA with AOT in isooctane and measured with NIPAB.
Modification of lyophilized wild-type PA with the cofactor in organic solvents

In anhydrous or low-water-containing solvents, enzymes may exhibit different behavior such as greatly enhanced stability, radically altered substrate specificity and enantioselectivity, molecular memory, and the ability to catalyze unusual reactions. The solubility of substrates and products can often be increased in organic media. Covalently immobilized PA has been used to catalyze the acylation of L-TyrOEt with methyl phenylacetate in water-miscible solvents at controlled water activity (a_w) (Basso et al., 2001). Cross-linked PA aggregates have been applied in the synthesis of ampicillin from D-phenylglycine amide and 6-aminopenicillanic acid (6-APA) in organic solvents (Cao et al., 2000). Inspired by these applications of PA in organic synthesis in anhydrous or low-water-containing solvents, wild-type PA and ΔM142C PA were modified in anhydrous or low-water-containing solvents with the phosphite ligand. Because the stability of PA varies in different solvents (Azevedo et al., 1999), the effects of three important parameters, (1) water content, (2) hydrophobicity (as reflected by the logP value, where P is the partition coefficient of the solvent between n-octanol and water), and (3) polarity of the solvent (as reflected by dielectric constant ε and dipole moment μ) were investigated. The results of these modification experiments are listed Table 3.

Table 3. The modification of PA in organic solvents.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Temperature (°C)</th>
<th>Time (h)</th>
<th>Activity (U/mg)</th>
<th>( \frac{V_{\text{ligand}}}{V_{\text{control}}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetone</td>
<td>30</td>
<td>24</td>
<td>4.95</td>
<td>1.31</td>
</tr>
<tr>
<td>acetone+1% H₂O</td>
<td>30</td>
<td>24</td>
<td>1.43</td>
<td>0.21</td>
</tr>
<tr>
<td>tert-butanol</td>
<td>37</td>
<td>72</td>
<td>2.81</td>
<td>2.25</td>
</tr>
<tr>
<td>tert-butanol+1% H₂O</td>
<td>37</td>
<td>96</td>
<td>5.48</td>
<td>4.11</td>
</tr>
<tr>
<td>tert-butanol+5% H₂O</td>
<td>37</td>
<td>72</td>
<td>3.36</td>
<td>0.36</td>
</tr>
<tr>
<td>n-hexane</td>
<td>30</td>
<td>48</td>
<td>3.88</td>
<td>0.90</td>
</tr>
<tr>
<td>n-propanol</td>
<td>30</td>
<td>48</td>
<td>3.62</td>
<td>2.56</td>
</tr>
<tr>
<td>n-propanol+1% H₂O</td>
<td>30</td>
<td>48</td>
<td>5.73</td>
<td>4.22</td>
</tr>
<tr>
<td>toluene</td>
<td>30</td>
<td>24</td>
<td>0.61</td>
<td>0.42</td>
</tr>
<tr>
<td>toluene+98% KCl</td>
<td>30</td>
<td>24</td>
<td>3.12</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* During modification the vials contained 1.0 mg lyophilized PA suspended in 1.0 mL solvents. The phosphite ligand was added to 1.2 mg/mL and the mixture was shaken at 200 rpm for the time indicated.

b Rate of inactivation in the presence of ligand compared to rate of inactivation without ligand.

The best modification was observed in tert-butanol containing 5% water and in acetone containing 1% water, where over 85% of the enzyme activity was inhibited after incubation of the suspended enzyme with the ligand. However, most of the enzyme that was modified in this way could not be redissolved in 0.1 M MOPS (pH 7.0). It has been described that although bound water enhances the conformational flexibility of an enzyme in an organic solvent, which should promote catalysis, enzymes start to become inactivated beyond a threshold of water concentration because the hydrophobic core of the enzyme is exposed to the solvent and the tertiary structure becomes disrupted (Zaks and Klibanov, 1988). Among the solvents tested, wild-type PA was inhibited by the ligand relatively efficiently in acetone (74%
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redaction of enzyme activity after 24 h) and more protein could be recovered in MOPS (pH 7.0) than in case modification was carried out under the conditions mentioned above. Therefore, acetone was chosen as the solvent for the modification of PA with the ligand.

Hydrogenation reactions catalyzed by PA-Phos-Rh
After modification of PA with the phosphite ligand in acetone, the original enzyme activity was inhibited. Complexation of the non-protein moiety with a metal precursor was attempted to generate a metalloprotein that can act as a hybrid catalyst. Although rhodium is assumed to preferentially bind to the phosphite ligand rather than to the side chains of amino acids or the backbone of the protein, the possibility of unspecific binding could not be excluded. According to previous studies, histidine, methionine, cysteine and to some extent tryptophan are likely to be residues that may compete with the ligand for metal binding (Giraldi et al., 1978; Katsaros et al., 2002; Trynda-Lemiesz et al., 1995). In view of the presence of such residues in wild-type PA, it was assumed that up to 59 equivalents of Rh(I) could possibly complex to the protein. Therefore, to achieve complete complexation, the rhodium precursor should be added in excess. On the other hand, an excess of rhodium could mask or even dominate the activity and selectivity of the rhodium bound by the phosphite ligand in the hydrogenation reactions catalyzed by the hybrid enzyme.

In order to prevent an effect of unspecifically bound rhodium, two methods were used for the complexation of PA with Rh(COD)2BF4. In the first method, the lyophilized wild-type PA (7.0-13.0 mg) was suspended in acetone (1.0 mL) and modified with 25- to 50-fold equivalents of ligand. After incubation at 37°C with shaking at 200 rpm for 20 h, the enzyme powder was collected by centrifugation and washed with n-propanol 3 times. Then, water was added to the enzyme powder and about 0.9-1.0 mg of enzyme was dissolved as judged by a protein assay. The modified PA (PA-Phos) (10.0 μM) was then complexed with Rh(COD)2BF4 (0.08-0.49 mM) in water. After removal of the excess Rh by gel filtration, the metallo PA-Phos (PA-Phos-Rh) was tested as a catalyst for the hydrogenation of methyl 2-acetamidoacrylate (Scheme 1). Hydrogenation reactions were done by Dr. Panella. For the control experiment, PA was complexed with 1-21 equivalents of Rh(COD)2BF4. After purification by gel filtration, the metalloprotein (PA-Rh) was used for the hydrogenation reaction.

Scheme 1. The Rh-catalyzed hydrogenation of methyl 2-(acetamido)acrylate.

In the second method, the ligand (3.7 mg) was complexed with Rh(COD)2BF4 (0.8-1.7 mg) in a ratio of 2:1 or 1:1 in acetone (1.0 mL) and then the complex (Phos-Rh) (4 μmoles) was added to wild-type PA (0.1 μmole) suspended in acetone (1.0 mL). The modification of PA with the cofactor was confirmed by checking the enzyme activity against NIPAB. After the modification, the semisynthetic metalloenzyme (PA-Phos-Rh) was collected in a centrifuge and washed with n-
propanol for 3 times to remove the unbound cofactor. A control sample (PA-Rh) was prepared without modification with the phosphite ligand.

The hydrogenation of methyl 2-acetamidoacrylate (0.08-0.2 mmole) was either performed in \( n \)-propanol (heterogeneously) or in phosphate buffer (pH 7.0) (homogeneously). The results of hydrogenation reactions are listed in Table 3. Both modification methods gave enzyme that was capable of full conversion of the substrate. PA-Phos-Rh was catalytically active and 100% conversion of the substrate was observed after 12 h. The control sample (PA-Rh) was much less active than PA-Phos-Rh and only 22% - 29% substrate was converted to the product by PA-Rh. However, no e.e. of the formed product was observed using the hybrid catalyst prepared by either method.

Table 4. The results of hydrogenation of methyl 2-(acetamido)acrylate catalyzed by PA-Phos-Rh. Entries 1 and 4, coupling of phosphite ligand to PA prior to complexation with Rh; entries 2 and 3, coupling of preformed phosphite-Rh complex to PA.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Rh:PA</th>
<th>Medium</th>
<th>Conversion (%)</th>
<th>TON</th>
<th>e.e. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+ligand 49:1</td>
<td>phosphate buffer</td>
<td>100</td>
<td>800</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-ligand 21:1</td>
<td></td>
<td>28.7</td>
<td>230</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>+ligand 1:1 (Rh:phosphate)</td>
<td>phosphate buffer</td>
<td>100</td>
<td>2000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-ligand 8:1</td>
<td></td>
<td>26</td>
<td>520</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>+ligand 1:2 (Rh:phosphate)</td>
<td>n-propanol</td>
<td>100</td>
<td>2000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-ligand 1:2</td>
<td></td>
<td>22.3</td>
<td>446</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>+ligand 1:1</td>
<td>phosphate buffer</td>
<td>99.3%</td>
<td>794</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-ligand 1:1</td>
<td></td>
<td>n.d. a</td>
<td>n.d. b</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a The \( H_2 \) pressure was 12 bar. The amount of substrate was 0.08 mmol (entry 1-2) or 0.2 mmol (entry 3-4). The amount of enzyme was 0.1 \( \mu \)mol. Measurements by L. Panella as described (Panella et al., 2005; 2006).

b n.d., not detectable.

For the first complexation method, the result of the hydrogenation catalyzed by the protein without ligand (PA-Rh) complexed with 8 equivalent Rh(I) was similar to that by PA-Rh complexed with 21 equivalents of Rh(I). This means that 8 equivalents of Rh(I) could well be an excess since many of the potential rhodium-binding residues are buried in the structure and may not be accessible. The results indicate that there are unspecific binding sites for rhodium in PA but the rate of the reaction catalyzed by metallic rhodium is much lower than the ligand-bound rhodium-catalyzed reaction. Therefore, the aspecifically bound rhodium does not have a large influence on the enantioselectivity of the catalysis.

For the second complexation method, in which rhodium is pre-reacted with ligand prior to coupling to PA, the influence of the rhodium not bound to the ligand but bound to other sites of PA was excluded. In this case, Rh(COD)BF\(_4\) was first complexed with one equivalent amount of cofactor in acetone and then the Phos-Rh complex was attached to the active site of wild-type PA. The reaction was performed both in \( n \)-propanol and in phosphate buffer, but the product was still racemic.
Discussion

In this Chapter, we describe the construction of a semi-synthetic metalloenzyme by covalent modification of PA with a phosphite ligand and further complexation with Rh(COD)\(_2\)BF\(_4\). The catalyst thus obtained was active in the hydrogenation of 2-(acetamido)acrylate but showed no enantioselectivity. In the process of the construction of the PA-Phos-Rh hybrid catalyst, the most difficult problem encountered was the modification of the enzyme with the phosphite ligand. The modification of wild-type PA and \(\delta M142C\) PA with phenacyl bromide, the linker of the cofactor, is a fast process because of the high affinity of phenacyl bromide to the acyl binding site of PA and moderate solubility in aqueous solutions. However, the modification with the phosphite ligand was very slow due to the difficult access of the cofactor to the enzyme active site and the poor solubility of the cofactor in aqueous solutions. A number of remedies were attempted: a) using cosolvents, b) partial unfolding of the enzyme, c) modification in organic solvents.

Because PA was not so reactive to the phosphate ligand as papain, the concentration of enzyme (10 \(\mu\)M) and the ligand (0.2-0.4 mM) were much higher than those (0.12 \(\mu\)M and 12 \(\mu\)M respectively) applied in the modification of papain. Under such conditions, the solubilization of the phosphite ligand became a serious problem. The addition of cosolvents improved the solubilization of the ligand in aqueous medium but had a negative effect on enzyme stability. In order to obtain a clear solution of 0.2 mM ligand in phosphate buffer, the solvents needed to be added to more than 80% which was not an applicable concentration for the enzyme, except in case of glycerol. PA was found stable in glycerol (80%), 1,2-propanediol (30%), DMSO (10%) and acetone (40%) for 6 h without significant loss of activity. However, in 80% glycerol, no inhibition of PA (10 \(\mu\)M) activity was observed when the ligand (0.4 mM) was added. The reason might be the bulky size of the phosphate ligand which possibly did not enter the active. Alternatively, the extremely high viscosity of glycerol could have prevented mass transfer of the ligand (Castro and Knubovets, 2003). In 40% acetone containing 0.4 mM ligand, 60% of PA (10 \(\mu\)M) activity was inhibited by the ligand, but this only happened in a reaction volume of 25 \(\mu\)L. If the reaction volume was increased to 1.0 mL, no inhibition of PA activity was observed. It could be concluded that the active site of PA is not sufficiently accessible for the bulky cofactor. However, if this is true it is difficult to explain the different behaviors of PA in different reaction volumes (25 \(\mu\)L-1.0 mL) when acetone was used as the cosolvent and it may be that glycerol prevents inactivation by some other mechanism.

In order to know whether the phosphite ligand was still good, we tested the modification of papain and flavodoxin which have cysteine residues that are more accessible to the bulk solvent. After the treatment with the phosphite ligand from the same batch as used in PA, both papain and flavodoxin were proved to be modified with the ligand. This result indicated that the difficulty in the modification of PA with the phosphite ligand was due to the steric hindrance caused the active site of PA which is much narrower than that of papain, even though the docking results showed that the ligand should be able to bind in the active site with the reactive group very close to the nucleophilic serine. Partial unfolding of PA was tried to make the active site of PA more accessible to the phosphate ligand. In an investigation of the enantioselective reductive amination of \(\alpha\)-keto acids to \(\alpha\)-amino acids by pyridoxamine cofactor with intestinal fatty acid binding protein (IFABP), the IFABP
protein was modified in unfolded form and after refolding the ligand was entrapped in the cavity (Kuang et al., 1996; 1997). However, PA is a heterodimer and we observed that it could not be refolded back to its natural conformation after unfolding. Some attempts were made to modify PA under partially unfolding conditions, for example using 1-3 M urea and 1 M guanidine hydrochloride. However, the enzyme incubated with and without the ligand showed the same decrease of activity under these slightly denaturing conditions. Therefore, the modification of PA with the ligand under denaturing conditions in aqueous solutions with cosolvents was concluded to be not successful.

In order to overcome the solubility problem of the ligand in aqueous solutions, modification of PA was explored in organic solvents. Different methods were used including 1) solubilizing PA in organic solvents by making reversed micelles or ion pairing with a surfactant, and 2) using a suspension of the lyophilized or n-propanol precipitated PA in organic solvents. PA was solubilized by reversed micelles of AOT in isoctane and was active in organic media (Kabakov et al. 1991, 1992, 1994, 1995; Alves et al., 1995). We explored this strategy for the modification of PA with the synthetic cofactor. Although as much as 28% of the added PA could be extracted to isoctane using reversed micelles of AOT, no inhibition of the activity was observed when the ligand was added to isoctane. The lack of the modification of PA in the reversed micelles in isoctane again suggests that the active site of PA is poorly accessible to the ligand.

For the use of a suspension of lyophilized or n-propanol precipitated PA, the choice of solvents was based on their hydrophobicity and polarity. Enzymes are expected to be more rigid in hydrophobic solvents that have a lower tendency to strip water from proteins than in hydrophilic solvents (Gupta and Roy, 2004). On the other hand, enzymes should be more flexible in more polar solvents (Narayan and Klibanov, 1993). Of the solvents tested, acetone has a moderate hydrophobicity ($\log P = -0.23$) and relatively high polarity (dielectric constant $\varepsilon = 20.7$, dipole moment $\mu = 2.69$). Toluene and tert-butanol are more hydrophobic and more apolar than acetone ($\log P = 0.8$, dielectric constant $\varepsilon = 12.5$, dipole moment $\mu = 1.69$ for tert-butanol and $\log P = 2.5$, dielectric constant $\varepsilon = 2.38$, dipole moment $\mu = 0.30$ for toluene). In glycerol, the lyophilized enzyme could be fully dissolved due to the solvents’ low hydrophobicity ($\log P = -3.0$) and high polarity ($\varepsilon = 42.5$), but the half-life of PA in glycerol is only about 4 h, as observed in this study. In the end, acetone was chosen as the solvent for the modification. When the lyophilized PA was suspended in acetone containing the ligand, about 74% of the activity was inhibited compared to the activity of the suspension without the ligand. The addition of a small amount of water (1-5%) to the solvents facilitated the modification, probably because hydration which allows enzymes to exhibit the conformational mobility required for reactions to occur (Klibanov, 1997). However, hydration also causes the enzymes to be less rigid in the solvents and thus can cause inactivation of the enzyme.

When PA-Phos-Rh was used in the hydrogenation of methyl 2-(acetamido)acrylate, complete conversion of the substrate was observed. These results show that a new hybrid catalyst could be obtained by modifying PA with an artificial cofactor. The modification of $\beta$Ser1 in the active site of PA with the phosphite ligand inhibited most of the original enzyme activity and after complexation with rhodium the semi-synthetic enzyme was catalytically active for hydrogenation reactions. However, no enantioselectivity was observed in the reaction. In order to find out whether the racemate was produced by rhodium complexed to the ligand or
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Rhodium bound at other sites instead of the phosphite ligand, unliganded PA was treated with Rh(COD)2BF4, and PA-Rh thus obtained was used to catalyze the hydrogenation. The conversion of 2-(acetamido)acrylate by PA-Rh was 22 – 29%, indicating that there was some rhodium bound in PA. Since the conversion of the substrate by PA-Rh was not dominant compared to PA-Phos-Rh, the activity and enantioselectivity of the catalyst were mainly dependent on rhodium complexed to the phosphite ligand. Although no direct clue for modification of the active site serine of PA was obtained by MS, the fact that PA-Phos-Rh was much more active than PA-Rh in hydrogenation reactions somehow confirmed that PA-Phos-Rh contained the phosphite ligand.

The cause of the lack of enantioselectivity of the catalyst might be that there are no interactions between the cofactor and the protein scaffold apart from the covalent linkage with the effect that the coordination geometries of the metal center could not be tuned by the protein scaffold. If the metal center coordinated by a ligand is surrounded by a protein, the protein should induce axial chirality in the ligand which is flexible and can assume two different configurations. So far we can conclude that no ‘enzyme-like’ interactions have occurred in our hybrid PAs that exert a differential influence, and that no chirality is transferred to the product. The observation that this occurs in a protein with a rather buried anchoring position, that is even inaccessible under many conditions, indicates that finding the right protein scaffold for incorporating the ligand and rhodium may be a critical step in developing such hybrid enzymes.

For future studies, directed evolution is a strategy to modify the protein environment and introduce additional interactions. Compared to papain from papaya, PA is a better candidate for directed evolution because it can be easily produced in E. coli and is self-processed to the mature form. However, cofactors with improved solubility in aqueous solutions should be prepared since this will be essential for screening libraries of mutants. The hybrid catalysts can be expected to display different activities and selectivities in a given reaction, depending on the nature of the mutant protein that surrounds the synthetic group (Reetz, 2004; 2006).

Another option to obtain a suitable protein scaffold for the artificial cofactor is borrowed from the concept of catalytic antibodies (abzymes) (Lerner et al., 1991). Catalytic antibodies are produced through immunization with a hapten molecule that is usually designed to resemble the transition-state intermediate of a desired reaction. They can force the substrates into transition-state geometry and stabilize the transition-state intermediates as enzymes do in the catalysis. The incorporation of metallic cofactors into catalytic antibodies has been investigated (Ersoy et al., 1996; Finn et al., 1998; Tanaka et al., 1999; Nicholas et al., 2002). An antibody elicited against a tin (IV) phorphyrin showed the desired oxidative activity upon assembly with a ruthenium (II) cofactor, transferring oxygen to thioanisole (Nimri and Keinan, 1999). Considering the difficulties in searching for suitable protein candidates for the hybrid catalyst within known enzymes, catalytic antibodies are good alternatives. If the complexes of cofactors and transition-state analogs are used as haptenens, it should be possible to screen the antibodies with the desired activity and enantioselectivity especially when high-throughput screening and immunological techniques such as ELISA are combined. This way tailored proteins for the cofactor and the substrate could be obtained.
References

Hydrogenation catalyzed by an artificial metalloenzyme based on penicillin acylase