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Multicore Artificial Metalloenzymes derived from acylated proteins as catalysts for the enantioselective dihydroxylation and epoxidation of styrene derivatives.

Melanie Leurs[a], Bjoern Dorn[a], Sascha Wilhelm[a], Magiliny Manisegaran[a] and Joerg. C. Tiller*[a]

Abstract: Artificial metalloenzymes (AME's) are an interesting class of selective catalysts, where the chiral environment of proteins is used as chiral ligand for a catalytic metal. Commonly, the active site of an enzyme is modified with a catalytically active metal. Here we present an approach, where the commercial proteins lysozyme (LYS) and bovine serum albumin (BSA) can be converted into highly active and enantioselective AME's. This is achieved by acylation of the proteins primary amino groups, which affords the metal salts in the core of the protein. A series of differently acylated LYS and BSA were reacted with $K_2OsO_2(OH)_4$, $RuCl_3$, and $Ti(OMe)_4$, respectively, and the conjugates were tested for their catalytic activity in dihydroxylation and epoxidation of styrene and its derivatives. The best suited system for dihydroxylation is fully acetylated LYS conjugated with $K_2OsO_2(OH)_4$, which converts styrene to 1,2-phenylethanediol with an enantioselectivity of 95 %ee *(S)*. BSA fully acylated with hexanoic acid and conjugated with three mol $RuCl_3$ per mol protein shows the highest ee values for the conversion of styrene to the respective epoxide with enenatioselectivities of over 80 %ee *(R)*, a TON of more than 2500 and a yield of up to 78 % within 24 h at 40 °C. LYS has two favored selective binding sites for the metal catalyst and BSA has even three. The AME's with titanate in the active center invert the enantioselectivity of styrene epoxidation.

Introduction

Enzymes have been established as highly selective and active catalysts in water, but also in organic solvents. The biocatalysts are used in fields such as biosensors and production of fine chemicals.[1] In general, the naturally limited substrate spectrum and activity of enzymes in organic solvents has been improved by several techniques including protein engineering[2], medium engineering[3], and binding on suitable supports.[4] The latter has been explored in our group, by using amphiphilic conetworks as films[4e, 4f, 5] and particles[4h, 6] or by applying electrospun nanofibers[4g] as enzyme activating matrices.

In order to widen the diversity of enzyme catalyzed reactions the active sites of enzymes were modified with alternative metals. This led to so-called artificial metalloenzymes (AME's), which combine the molecular recognition ability of proteins with the broad reactivity scope of small molecule catalysts.[7] Kaiser and co-workers were the first to generate such a hybrid catalyst by replacing the natural zinc of carboxypeptidase A by copper and they used the resulting AME in oxidation reactions.[8] AME's, generated by different anchoring strategies, have been applied in various types of chemical reactions[7k], for example, imine reduction[7g], sulfoxidation[9], hydrogenation[10], Diels-Alder reactions[11], diastereoselective coenzyme reduction[12], dihydroxylation[13] or epoxidation.[13d, 14]

Most of the reactions catalyzed by AME's are carried out in aqueous media, but there are also some examples, which involve organic co-solvents.[7c, 7d, 14b, 15]

A multitude of these reactions can be performed with natural enzymes, too, which are in many cases much more active but often less stable as artificial enzymes particularly in aqueous/organic co-solvent systems.

Recently, we have shown that the conjugation of enzymes with polymers, such as poly(2-methyl-2-oxazoline) (PMOx) renders the biocatalysts soluble in organic media with improved activity compared to the native enzyme powder.[16] The organo-soluble conjugates were also used as core-shell nanocontainers to drive organo-insoluble salts into the enzyme structure that forces the heavy metal ions to bind in the chiral environment of the enzyme, possibly in the active site. This leads to the generation of organo-soluble AME's. Those are active in the dihydroxylation of styrene in chloroform, which affords in the case of laccase as enzyme enantioselectivities of the product formation of more than 99 %ee (*(R)*-enantiomer).[17] However, the method of preparing these conjugates is rather tedious and the used enzymes are expensive. Besides making the protein organo-soluble, the major role of the conjugation might be blocking the primary, easily accessible amino groups in the protein scaffold. This way, the heavy metals are forced to bind in less accessible sites of the protein, which are more likely forming a chiral environment than binding positions rather at the surface of the biomacromolecule.

In the present study, we explore this concept of blocking the amino groups of readily available proteins by acylation and rendering them into AME's active in oxidation reactions.

[a] Dipl.-Chem. Melanie Leurs, M.Sc. Bjoern Dorn, M.Sc. Sascha Wilhelm, B.Sc. Magiliny Manisegaran, Prof. Dr. rer nat. Joerg C. Tiller*
Chair of Biomaterials and Polymer Science, Department of Biochemical and Chemical Engineering, TU Dortmund, Emil-Figge-Str. 66, 44227 Dortmund, Germany
* To whom correspondence should be addressed. Phone: +49 (0)231 755 2479, Fax: +49 (0)231 755 2480, Email: joerg.tiller@tu-dortmund.de

Supporting information for this article is given via a link at the end of the document.

Results and Discussion

The objective of this work was to render inexpensive proteins into AME's for the enantioselective synthesis in organic solvents. The rational is that blocking the primary amino groups by acylation might block the outer non-chiral binding sites, mostly the lysine-based primary amino groups, for heavy metal catalysts and force those into less accessible, but likely better chirality controlling sites of the protein. (see Figure 1).

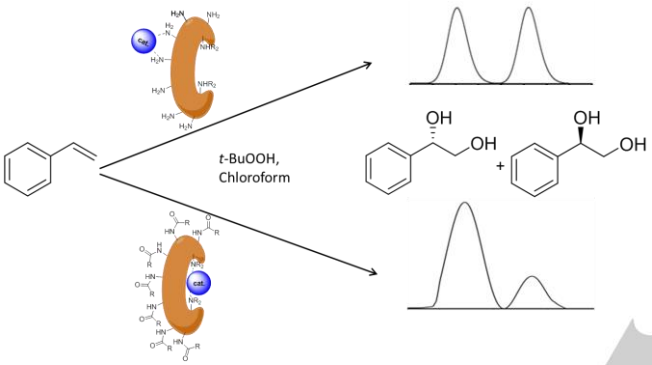

Figure 1. Concept of acylated artificial metallo enzymes for asymmetric dihydroxylation.

Lysozyme (LYS) and bovine serum albumin (BSA) were chosen as readily available inexpensive and stable proteins. They were modified with different acyl anhydrides according to established literature protocols for N-acylation.[18] First the results of LYS acylation are discussed. The conversion was followed by photometric titration of the remaining primary amino groups. As seen in Figure 2, the conversion of the amino groups strongly depends on the excess of acyl anhydride, providing the opportunity to control the degree of modification. In general, the acetylation (AA) seems to be less efficient than propionylation (PA) and hexanoylation (HA). To verify the selectivity of the acylation reaction for primary amine groups, unmodified LYS and LYS-AA were pH-titrated between pH 3.3 and 11.6 (see Figure S1) according to a literature protocol.[19] The given plot displays the number of titratable groups in the chosen pH range. Comparison of the final values at pH 11.6 shows 28 titratable groups for LYS und 22 for LYS-AA, which is in good agreement with the expectations for the acetylation of the 6 primary amine groups of the lysine units. Furthermore this experiment strongly indicates that no titratable functional group other than the primary amine groups was modified during the acylation.

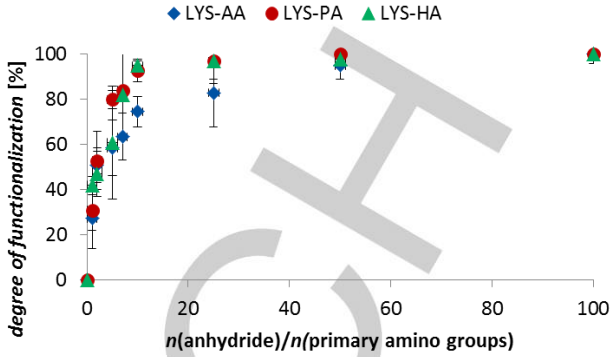

Figure 2. Degree of functionalization vs. molar equivalents of anhydride per primary amino group of enzyme plot for the functionalization of lysozyme (LYS). The degree of functionalization was measured spectrophotometrically by an adjusted TNBS-Assay according to literature known protocols[20] (see experimental section).

The resulting acylated LYS's are not soluble in organic solvents in contrast to the respective formerly reported poly(2-oxazoline) (POx)-conjugates[16c, 17a], but form stable suspensions in various organic solvents such as toluene and chloroform (Figure 3).

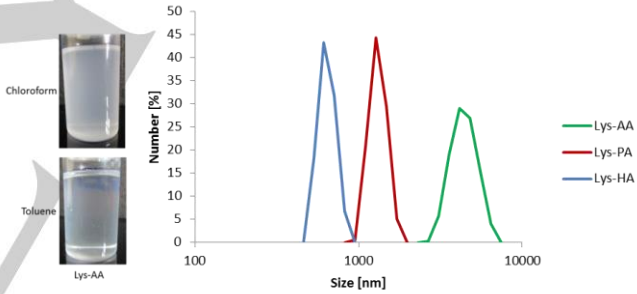

Figure 3. Pictures of suspensions of 4.2 mg fully acetylated LYS (LYS-AA) in 10 mL chloroform (left, top) and toluene (left, bottom) and DLS measurements of the fully acylated LYS in toluene (right).

DLS measurements of the acylated LYS in toluene revealed that the size of the formed aggregates decreases in the order AA (4-6 µm)>PA (1-2 µm)>HA (0.5-0.8 µm) (Figure 3, right). This is in good compliance with the expectation that nonpolar solvents such as toluene should afford finer dispersions of the conjugates because the modification inserts an increasingly less polar carbon chain to the protein. No particles could be detected by DLS in the chloroform suspensions, indicating particle sizes larger than 10 µm.

First, the established dihydroxylation of styrene was carried out with a 1:1 (mol/mol) osmate complex with the modified lysozymes (Os-LYS, metal to protein ratio (MPR) = 1). It is known to literature, that enzyme activity in organic solvents strongly depends on the protonation state of the protein.[3b] The pH can be adjusted in aqueous environment and the resulting

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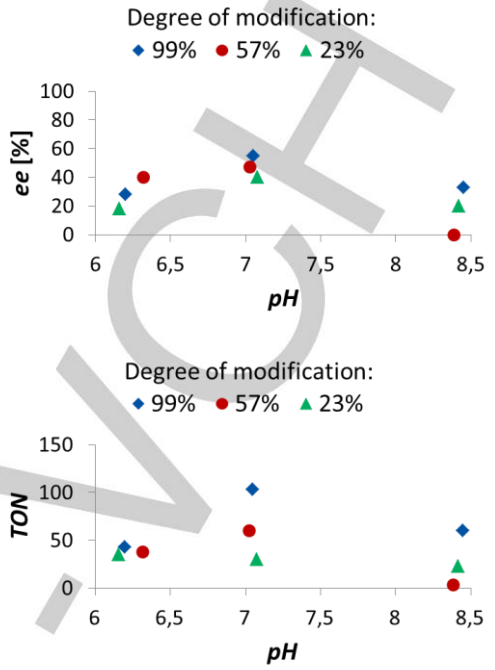
protonation state remains frozen in organic media (pH-memory).[3b] This way, the acetylated LYS with varying degrees of modification were adjusted to three different pH values (pH 6.3, 7.0, and 8.4) prior to lyophilization. Then, the acetylated as well as the unmodified LYS's were suspended in chloroform, potassium osmate was added, and after 30 min of stirring styrene and peroxide were given to the suspension. The product was isolated after 7 d at 0 °C and analyzed by HPLC. No product formation was found for the suspended pristine enzyme powder. In contrast, Os-LYS-AA affords a significant formation of 1,2-phenylethanediol. All derivatives favor synthesis of the *(S)*-enantiomer of the product (Fig. 4).

As seen in Figure 4 a), the highest activity and selectivity was achieved at pH 7. The higher the number of modified amino groups, the higher was the selectivity and also the activity. This proves the concept of this work to be effective, i.e. the blocking of the amino groups of proteins forces the catalytic metal into regions of the protein that better control the enantioselectivity and also the activity.

a)



b)

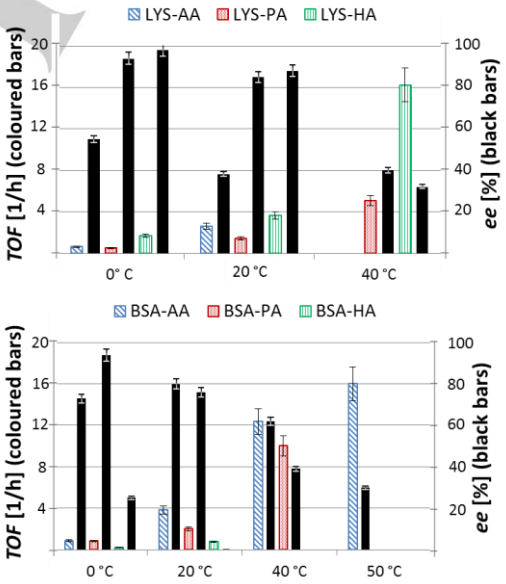


Figure 4: a): Turnover number (TON) and enantiomeric excess (*ee, (S)*-enantiomer) vs. pH plot of the asymmetric dihydroxylation catalyzed by Os-LYS-AA with an MPR of 1. The reaction was carried out with 0.03 µmol/mL LYS-AA, 0.03 µmol/mL $K_2OsO_2(OH)_4$, 110 µmol/mL tBuOOH and 100 µmol/mL styrene at 0°C for 7 d in chloroform. The TON is defined as µmol product per µmol protein. The degree of modification is the percentage of primary amines of the protein blocked by acylation.
b): Comparison of turnover frequency (TOF, coloured bars) and enantiomeric excess (*ee, (S)*-enantiomer, black bars) of the asymmetric dihydroxylation catalyzed by osmate based AME's (pH 7, fully acylated) with an MPR of 1 at different temperatures. The reaction was carried out with 0.03 µmol/mL protein conjugate, 0.03 µmol/mL $K_2OsO_2(OH)_4$, 110 µmol/mL tBuOOH and 100 µmol/mL styrene. The reaction times were adjusted to previously determined surviving times of a respective Os-laccase-PMOx under the used conditions and temperatures, being 7 d at 0 °C, 72 h at 20 °C, and 24 h at 40 °C.[17a] The TOF is defined as µmol product per µmol protein per hour.

All further investigations were carried out with fully acylated proteins adjusted to pH 7. The different acylated LYS's were conjugated with osmate and the catalytic activity for the dihydroxylation of styrene was explored at different temperatures.

Taking a look at Figure 4 b), top, shows that the best enantioselectivity in the dihydroxylation of styrene with lysozyme based AME's was achieved with some 98 %ee *(S)* by using Os-LYS-HA at 0 °C. The respective Os-LYS-PA also affords a high enantioselectivity with 95 %ee *(S)* under the same reaction conditions but about 4-fold lower activity. In comparison to the latter Os-LYS-AA shows the lowest enantioselectivity with 57 %ee *(S)* and also a lower activity. Increasing the temperature to 20 °C expectedly increases the activity by a factor of 2-5 and lowers the enenatioselectivity. Os-LYS-HA and OS-LYS-PA retain enenatioselectivities of 88 and 85 %ee *(S)*, respectively. Further increasing the reaction temperature to 40 °C leads to greatly enhanced activities, but the enantioselectivity drops to below 40 %ee *(S)* in both cases.

The same experiments were carried out with fully acylated BSA (Figure 4 b), bottom). The optimal pH for high activity and selectivity was found to be 7.0 as well. Os-BSA does catalyze styrene dihydroxylation. Os-BSA-AA, however, shows the highest activity (TOF 0.9 h^{-1}, TON of 275 after 7 d at 0 °C) and also a high enantioselectivity (73 %ee *(S)*), favoring formation of the *(S)*-enantiomer. In case of Os-BSA-HA the product formation is hardly selective (25 %ee *(S)* at 0 °C), while Os-BSA-PA affords an enantioselectivity of 94 %ee *(S)* at 0 °C. Increasing the temperature to 20 °C also leads to increased TOF values and in the cases of Os-BSA-PA and Os-BSA-AA, the enantioselectivity is still high in a range of 76 – 80 %ee *(S)* at 20 °C. Remarkably, Os-BSA-AA shows a selectivity which is higher than 60 %ee *(S)* at 40 °C.

Altogether, the measured selectivities of the 1:1 complexes of acylated LYS and BSA with osmate are with up to 98 %ee *(S)* comparable to that of other designed AME's.[13a, 17b] This shows that simple acylation of readily available proteins and complexation with osmate renders those into enantioselective metalloenzymes for the dihydroxylation of styrene in organic solvents. The enantioselectivity depends on the nature of the protein and the protein specific choice of the carbonic anhydride used for modification. BSA is more stable with respect of retaining enantioselectivity at higher temperatures.

As shown previously, the solvent plays an important role in the selectivity of enzymes. [17a] This was addressed by carrying out the experiments above at 0 °C with acylated LYS's in toluene (results provided in Table S2 in the supplements). The activity of all three conjugates is similar to that of the most active AME Os-LYS-AA in chloroform, but the enantioselectivity is below 30 %ee favoring the *(S)*-enantiomer in all cases. This confirms the great influence of solvents on stereoselective reactions of even artificial metalloenzymes.

A number of alkenes were investigated as substrates for the most active and selective AME Os-LYS-HA in chloroform at 20 °C. The results, summarized in Table 1, show that this complex catalyzes the dihydroxylation of 4-chlorostyrene, 4-methylstyrene and α-methylstyrene. In case of 4-chlorostyrene the achieved TON is about 2 times lower than that for styrene and also the enantioselectivity is with 50 %ee *(S)* significantly lower. The substrates α-methylstyrene and 4-methylstyrene are converted with lower rates and the product formation cannot be considered enantioselective. 1H-Indene and allylphenylether are not converted at all. Investing the conversion of these substrates with Os-BSA-AA leads to a similar picture, with exception of 4-methylstyrene, which is converted with a 5-fold higher TON and the *(S)*-enantiomer is favored with an enantioselectivity of 90 %ee *(S)*. The Os-BSA-AA is also not active in the conversion of 1H-Indene and allylphenylether. The high substrate selectivity of the acylated protein complexes might be usable for separation of product mixtures of styrene derivatives. The previously reported Os-laccase-PMOx shows a different substrate selectivity, e.g., catalyzing the dihydroxylation of 1H Indene (TON = 5.2 after 7 d at 0 °C, 71 %ee *(1R,2S)*) and allylphenylether (TON = 1.2 after 7 d at 0 °C, 87 %ee *(R)*) but no conversion was found for 4-methylstyrene and α-methylstyrene. This underlines the strong role of the nature of protein for the catalytic process of AME's.

Table 1: Comparison of turnover frequency (TOF), yield, turnover number (TON) and enantiomeric excess (*ee*) of the asymmetric dihydroxylation catalyzed by Osmate-based AME's (pH 7, fully acylated) with an MPR of 1 with different alkenes at 20 °C for 3 d. The reaction was carried out with 0.03 µmol/mL protein conjugate, 0.03 µmol/mL $K_2OsO_2(OH)_4$, 110 µmol/mL tBuOOH and 100 µmol/mL alkene for 3 d in chloroform. The TON is defined as µmol product per µmol protein. The TOF is defined as µmol product per µmol protein per hour. [1]H-Indene and allylphenylether are not converted by both AME's.

AME	alkene	*ee* [%]	TON	TOF [h^{-1}]	Yield [%]
Os-LYS-HA	4-chlorostyrene	50	112	1.5	3.4
Os-LYS-HA	4-methylstyrene	43	20	0.3	0.6
Os-LYS-HA	α-methylstyrene	31	3	0.04	0.09
Os-BSA-AA	4-chlorostyrene	51	55	0.8	1.7
Os-BSA-AA	4-methylstyrene	90	108	1.5	3.3
Os.BSA-AA	α-methylstyrene	26	5	0.06	0.1

In order to broaden the concept of using AME's prepared by complexation of acylated proteins with metals, LYS and BSA were conjugated with catalysts suitable for epoxidation. $Ti(OMe)_4$, $Mn(Ac)_2$, and $RuCl_3$, respectively, were considered as potential candidates as suggested in the literature.[21] Fully acylated LYS and BSA, respectively, adjusted to pH 7.0 were reacted in 1:1 molar ratio with the respective metal compound. The reaction was carried out in dichloromethane (DCM). Preliminary investigations showed that the by far highest conversion of styrene was achieved when using $RuCl_3$ as catalytic metal salt. The results of ruthenium-based AME's derived from acylated LYS will be discussed first.

The kinetics of the styrene epoxidation catalyzed by Ru-LYS-AA was measured at 0 °C, 20 °C, and 40 °C, respectively. As seen in Figure 5, the TON increases linearly at 0 °C during the entire reaction time of 7 d leading to a TON of 500 which calculates to 16 % conversion of styrene to styrene oxide. Only small amounts of aldehyde byproducts were found indicating product selectivities higher than 90 %. The enantioselectivity of the product formation favoring the *(R)*-enantiomer is with 95 %ee exceptionally high. When increasing the reaction temperature to 20 °C the slope of the curve is steeper, but no further product is formed after three days. The achieved TON of 749 calculates to 22 % yield, but the enantioselectivity drops to 63 %ee *(R)*. The activity further increases at 40 °C. However, no significant conversion occurs after 24 h. The yield is again increased to 47 % with a drop of enantioselectivity to 50 %ee *(R)*. This indicates that the generated active site for epoxidation in the protein is somehow altered during the course of the reaction like it has been found for dihydroxylation of styrene with Os-laccase-PMOx in a previous study.[17a] It is worth noting that the enantioselectivity does not significantly change during the course of the reaction (see Table S3 in the supplements). All following reactions were stopped after 24 h at 40 °C, after 72 h at 20 °C, and after 7d at 0 °C, respectively.

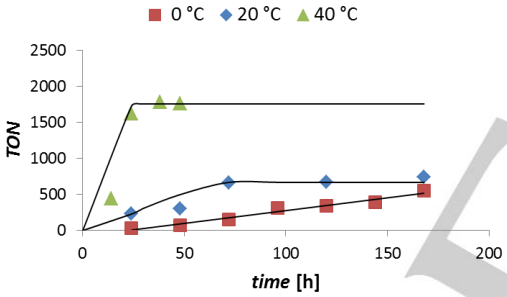


Figure 5: Turnover number (TON) vs. time plot for the asymmetric epoxidation catalyzed by Ru-LYS-AA (pH 7, MPR = 1) in DCM. The reaction was carried out with 0.06 µmol/mL LYS-AA, 0.06 µmol/mL $RuCl_3$, 220 µmol/mL tBuOOH and 200 µmol/mL styrene at 0 °C, 20 °C, and 40 °C, respectively. The TON is defined as µmol product per µmol protein. The black lines illustrate the trend of the data points. The enantioselectivity does not change in the curse of the reaction and the ee values are presented in Figure 6.

The styrene epoxidation catalyzed by the fully acylated Ru-LYS's was investigated at 20 °C. The activity of the AME's increases in the order LYS<LYS-HA< LYS-PA<LYS-AA (see Figure 6). The *(R)*-conformation of the formed epoxide is favored in all cases. The pristine Ru-LYS shows a high activity in epoxidizing styrene (TON = 361 after 3 d, TOF = 5.0 h^{-1}), but the product formation is hardly enantioselective (10 %ee *(R)*). The Ru-LYS-HA affords higher enantioselectivity of 56 %ee *(R)* with slightly increased activity (TOF = 6.2 h^{-1}, TON = 449 after 3 d) compared to Ru-LYS. Ru-LYS-PA (TON = 561, TOF = 7.7 h^{-1}) and Ru-LYS-AA (TON = 749, TOF = 10.4 h^{-1}) show a product selectivity of 74 and 63 %ee *(R)*, respectively, and even higher activities than Ru-LYS-HA. In the case of the styrene epoxidation catalyzed by Ru-LYS-AA an increase in temperature to 40 °C leads to a 6-fold increase in TOF and thus an epoxide yield of 46 % after 24 h with a selectivity of 50 %ee *(R)*. When raising the temperature of this reaction to 50 °C no increase in activity was observed, but the enatioselectivity was found to be only 30 %ee *(R)*.

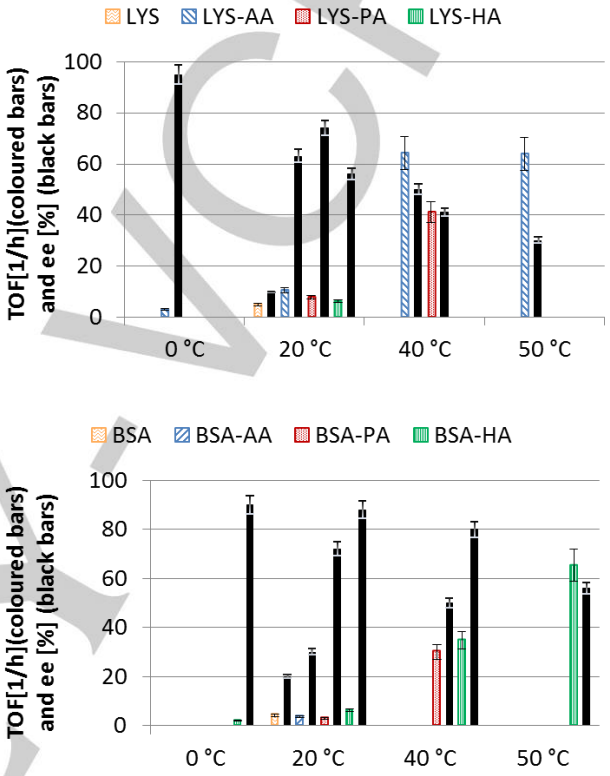


Figure 6: Comparison of turnover frequency (TOF, coloured bars) and enantiomeric excess (ee, *(R)*-enantiomer, black bars) for the asymmetric epoxidation catalyzed by Ru-based AME's (pH 7, MPR = 1) in DCM at different temperatures. The reaction was carried out with 0.06 µmol/mL protein, 0.06 µmol/mL $RuCl_3$, 220 µmol/mL tBuOOH and 200 µmol/mL styrene for 7 d at 0 °C, 72 h at 20 °C, and 24 h at 40 and 50 °C. The TOF is defined as µmol product per µmol protein per hour.

Carrying out the epoxidation of styrene catalyzed by acylated Ru-BSA AME's at 20°C reveals that the hexanoated Ru-BSA is the best suited epoxidation catalyst of styrene regarding ee (88 %ee *(R)*) and activity (TOF = 6 h^{-1}, TON = 452 after 3 d) (Figure 6). Ru-BSA-PA still affords an enantioselectivity of 72 %ee *(R)*, but the activity is less than half of that of Ru-BSA-HA. Epoxidation catalyzed by Ru-BSA-AA leads to product formation with only 30 %ee *(R)* and rather low activity. The pristine Ru-BSA also catalyzes epoxidation of styrene, but the product formation is hardly enantioselective resulting in an ee value of only 20 % for the *(R)*-enantiomer.

The TOF of Ru-BSA-HA increases nearly 6-fold when increasing the temperature from 20 °C to 40 °C. More remarkably, the selectivity is with 80%ee *(R)* still high. Further increasing the temperature to 50 °C allows 47 % conversion of styrene within

24 h, but the selectivity was found to be only 56 %ee *(R)*. Regarding the course of selectivity loss over temperature it can be concluded that the selectivity loss occurs at lower temperatures for the acylated Ru-LYS AME's than for respective Ru-BSA and thus the latter are more robust at higher temperatures.

Acylated LYS was also treated with $Mn(Ac)_2$. The resulting AME's were investigated as catalysts for the epoxidation of styrene at 20 °C for 3 d. The results are summarized in Table S4 in the supplements. The acylated Mn-LYS's show low TOFs of some 0.1 – 0.3 h^{-1} (TON = 8-24 after 3 d) and enantioselectivities similar to $RuCl_3$ based system. The activities are in the range of a literature known Mn-Carbonic Anhydrase (TON between 2.2 and 21 at 30 °C using BES buffer (0.1 M, pH 7.2) as reaction medium).[14c]

Alternatively, acetylated LYS's were treated with $Ti(OMe)_4$, which binds supposedly to the enzyme as titanate species, and investigated as catalysts for epoxidation of styrene at 20 °C for 3 d. The found TOF values are between 0.2 and 0.3 h^{-1} (TON = 14 -22 after 3 d) for Ti-LYS-PA and Ti-LYS-AA, respectively, and no conversion was found in the presence of the Ti-LYS-HA. Interestingly, the $Ti(OMe)_4$ based AME's favors the *(S)*-conformation of the product with remarkably high ee values of 86 and 90 %ee *(S)* for Ti-LYS-PA and Ti-LYS-AA, respectively. This inversion of stereoselectivity might be due to different mechanisms of the titanium and ruthenium-based catalysts, e.g. formation of metallacyclo intermediates of the acylated Ti-LYS and electrophilic oxidation in case of the acylated Ru-LYS.[22] Also the used tert-butylperoxide might play a role in this, because the titanium peroxide complexes form intermediates, which would then carry an additional ligand (tert-butyl), what might slow the reaction rate down and change the enantioselectivity. The Ti-BSA-HA also catalyzed the epoxidation of styrene with a slow rate but a fairly high enantioselectivity (75 %ee *(S)*). The design of the AME's was based on previous work, where the best enantioselectivities were achieved by 1:1 (molar ratio) complexes of protein (laccase) and metal compound (osmate). In order to explore, if the investigated proteins have more than one binding site that controls the enantioselectivity of the product formation, the metal salt $RuCl_3$ was added in varying ratios to LYS-AA and the reaction was carried out at 20 °C for 3 d. As seen in Figure 7, top, Ru_2-LYS-AA with two ruthenium centers is nearly doubling its activity, while slightly losing its enantioselectivity (65 %ee to 56 %ee *(R)*). The addition of one more ruthenium to the protein leads again to an increase in activity accompanied by a significant loss in enantioselectivity (35 %ee *(R)*). This means that the acetylated lysozyme contains two binding sites for ruthenium that enantioselectively catalyze the epoxidation of styrene to the *(R)*-enantiomer of the respective epoxide, i.e. it represents an AME with two active sites. There is also a third binding site that actively catalyzes this reaction but with no selectivity. Alternatively, it is thinkable that the excess of salt is only changing the equilibrium between metal salt and one active center that complexes the metal species. However, this seems less likely, because the salt is not soluble in the organic solvent. The same experiment was carried out with BSA-HA at 40 °C for 24 h. As seen in Figure 7, top, BSA-HA seems to contain even three selective binding sites for ruthenium. This Ru_3-BSA-HA is the most active and still highly enantioselective AME for the epoxidation of styrene. The reaction at 40 °C with only 0.18 µmol/L ruthenium chloride and 0.06 µmol/L BSA-HA leads to an epoxide yield of 78 % (TON = 2613, TOF = 109 h^{-1}) after 24 h and a high enantiomeric excess of 82 %ee *(R)*. This AME can compete with a recently reported ruthenium aqua complex supported on silica particles where an ee value of 79 % and a conversion of 92 % was found after 6 h for styrene at 25 °C using 1 mol% Ru.[21c] By increasing the MPR to 4 even a yield of 88 % can be achieved but the enantioselectivity decreases to 44 %ee *(R)*.

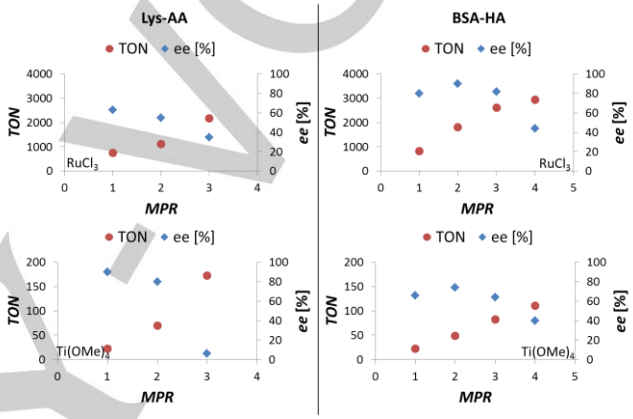


Figure 7: Metal to protein ratio (MPR) vs turnover number (TON) and enantiomeric excess (ee) plot for the asymmetric epoxidation of styrene catalyzed by Ru-LYS-AA and Ru-BSA-HA (top, *(R)*-enantiomer) and Ti-LYS-AA and Ru-BSA-HA (bottom, *(S)*-enantiomer) (PH 7) in DCM at 20 °C for 3 d for LYS-AA and at 40 °C for 24 h for BSA-HA. The reaction was carried out with 0.06 µmol/mL protein, 0.06 to 0.24 µmol/mL metal species, 220 µmol/mL tBuOOH and 200 µmol/mL styrene. The TON is defined as µmol product per µmol protein.

The same scenario was found for the Ti-LYS-AA, indicating that the titanate binds at similar positions as the ruthenium (Figure 7, bottom). The only difference is that the third binding site affords a larger drop in enantioselectivity than found for the respective Ru_3-LYS-HA. Comparison of these two AME's shows that the titanate based systems are more selective and less active than the respective ruthenium systems. Besides this difference, the binding sites of titanate and ruthenium seem to be similar in both acylated proteins.

Having established that the proteins might have more than one metal binding site, we investigated, if the thermally inactivated catalyst with one metal center can be re-activated by subsequent adding of another portion of metal species. If only the surrounding of the metal ion is oxidized, the additional metal ions might bind in other regions of the protein renewing the activity. Thus, the epoxidation of styrene was carried out with Ru-LYS-AA and Ru-BSA-HA, respectively, at 40 °C and another molar equivalent $RuCl_3$ was added to the reaction mixture after 24 h reaction time. The activity of both AME's could be renewed this way. Also subsequent lowering of the reaction temperature

has no re-activating effect on the AMEs. Thus we propose that the deactivation of the enzyme might be due to irreversible oxidation of the complexing groups in the protein or reaction with the formed styrene oxide with these groups.

To identify possible metal binding sites we took a deeper look at the protein structure (see Figures S5 and S6 in the supplements). The reaction with unmodified protein resulting in low ee values indicates that the easily accessible primary amino groups of lysine on the surface of the protein are possible binding sites of the metal ions. Blocking these groups by acylation leads to greatly increased enantioselectivities. Comparison with successful epoxidation catalyst ligands suggests that the secondary amines of tryptophan and histidine plus the tyrosine residues as potential docking sites. Lysozyme contains at least 3 tyrosines, 6 tryptophanes and 1 histidine.[23] Due to the high achieved ee values and thus the necessity of a stable binding in a chiral environment, it was assumed that the formed metal-protein complex leading to enantioselective product formation is at least bidental. For such a binding scenario we recognized three possible binding sites in lysozyme. Two of them (via Trp28, Tyr23 and Trp 108, Trp 111) are located at helical structures whereas the third one (Trp 62, Trp 63) is situated within a random coiled area of the protein (Figure S5). The fact that only two binding sites direct the stereoselectivity suggests that the best binding sites are in the region of the α-helices. BSA shows a more complicated structure containing 20 tyrosines, 2 tryptophanes and 17 histidines[24] and thus much more possible binding sites for metal compounds. Looking for opportunities to form bi- or multi-dental complexes metal binding also different opportunities are possible like it is, exemplarily, shown in Figure S6.

To broaden the reaction scope a number of styrene derivatives were investigated as substrates for Ru_3-BSA-HA and Ru_2-LYS-AA catalyzed epoxidation at 40 °C and 20 °C, respectively. The results, shown in Table 2, reveal that the reaction can also be carried out with 4-chlorostyrene, 4-methylstyrene and α-methylstyrene with significant conversion. 1H-Indene and allylphenylether are not converted. For example, 4-chlorostyrene is epoxidized by Ru_3-BSA-HA catalysis with 42 % yield and a high ee with 78 %ee *(R)*. The epoxidation of this substrate catalyzed by Ru_2-LYS-AA leads to an increase in enantioselectivity to 97 %ee *(R)* with 10 % yield. 4-methylstyrene is epoxidized to 21 % with a low enantioselectivity of 32 %ee of the *(R)*-enantiomer catalyzed by Ru_3-BSA-HA. In contrast, a high enantiomeric excess of 80 %ee *(R)* but a lower yield of 9 % is achieved by catalysis with Ru_2-LYS-AA of the epoxidation of the latter substrate. α-methylstyrene is converted to 24 % with a very high ee value for the *(R)*-enantiomer of 91 %ee *(R)* for when catalyzed by Ru_3-BSA-HA. In the case of Ru_2-LYS-AA a dramatic drop in both, conversion and ee was observed for this substrate. Thus, the efficiency of catalysis is strongly dependent on the nature of the protein and the styrene derivative. Also the spectrum for asymmetric epoxidations is broader than that of dihydroxylation catalyzed by osmate-based systems, but still limited to structures close to styrene.

Tab. 2: Comparison of turnover frequency (TOF), yield, turnover number (TON) and enantiomeric excess (*ee*) of the asymmetric epoxidation catalyzed by ruthenium based AME's (pH 7) at 40 °C for 24 h for Ru_3-BSA-HA and at 20 °C for 3 d for Ru_2-LYS-AA with different alkenes. The reaction was carried out with 0.06 protein, 0.18 and 0.12 µmol/mL µmol/mL $K_2OsO_2(OH)_4$, 220 µmol/mL tBuOOH and 200 µmol/mL alkene. The TON is defined as µmol product per µmol protein. The TOF is defined as µmol product per µmol protein per hour.

AME	alkene	*ee* [%]	TON	TOF [h^{-1}]	Yield [%]
Ru_3-BSA-HA	4-chlorostyrene	79	1381	58	42
Ru_3-BSA-HA	4-methylstyrene	36	703	29	21
Ru_3-BSA-HA	α-methylstyrene	91	789	33	24
Ru_2-LYS-AA	4-chlorostyrene	97	341	4.7	10
Ru_2-LYS-AA	4-methylstyrene	80	318	4.4	9
Ru_2-LYS-AA	α-methylstyrene	67	28	0.4	0.8

Conclusions

The concept idea of the present study was to render common proteins into highly selective AME's by blocking the primary amino groups by acylation. The chosen enzymes lysozyme and bovine serum albumin were acylated with different carboxylic acids and treated with $K_2OsO_2(OH)_4$, $Ti(OMe)_4$, and $RuCl_3$, respectively, in different organic solvents. It was found that acylation is required to obtain enantioselective dihydroxylation and epoxidation of styrene derivatives with AME's. This proves the concept to be valid and opens a great opportunity to readily achieve AME's as highly active and selective catalysts from natural sources. With respect to activity and selectivity, the acylated protein conjugates outperform literature known artificial metalloenzymes. For example, a xylanase equipped with a manganese porphyrin leads to a styrene oxide yield of 17 % and with an enantiomeric excess of 8.5 %ee *(S)* after 1 h at RT in phosphate buffer (50 mM, pH 7).[14b] Two other reports describe the epoxidation of different styrene derivatives by H_2O_2 as oxidant, catalyzed by bovine and human carbonic anhydrases, in which the original catalytic Zn^{2+} ion was replaced by a manganese species. The one published by Okrasa and Kazlauskas reports conversions between 1% (TON = 2.2 after 2 h) and 12 % (TON = 21 after 16 h), and enantiomeric excesses between 50 %ee and 66 %ee at 30 °C using BES buffer (0.1 M, pH 7.2) as reaction medium.[14c] Soumillion et al. used a phosphate buffer (50 mM, pH 7)/DMF mixture and reported yields between 9 % and 57 % with 18 % to 52 % ee overnight at 4 °C.[14a] Epoxidation of styrene with an artificial manganese chloroperoxidase from *Caldariomyces fumago* in BES buffer (0.1 M, pH 7.2) yields to 40 % after 5 h (TON 1500) with an enantiomeric excess of 49 %ee *(R)*.[14c] Also natural monooxygenases can be used for epoxidation reactions. For

example the usage of a fused StyA/StyB system from *pseudomonas* sp. leads to very high specific epoxidation activities for styrene of 95 min^{-1} (99 %ee *(S)* within the first 5 min (TON = 475).[25] Hollmann et al. reached a productivity of maximum 6.4 mM/h with 6.5 µM StyA after 15 min, what means a TOF of around 66 min^{-1}, and a total turnover number after 2 h of 662 with more than 98 %ee *(S)* for styrene epoxidation.[26] Thus the natural enzymes are much more active, but less stable than the artificial metalloenzymes used in this study.

It is particularly remarkable that the proteins used in this study might have more than one enantioselectivity controlling binding site. Lysozyme could contain two selective sites, and at least a third non-selective binding site. The BSA might have three selective sites and minimum a fourth that might direct to the opposite enantiomer. This shows that the protein structures are indeed rich of different chiral binding sites and there might be more opportunities to direct the catalytic metal to those, e.g. by blocking other functional groups or even introducing new and more efficient ones by chemical modification. Introducing metal complexing functional groups that are less susceptible for oxidation or alkylation might make the AMEs more stable and possible more active.

Experimental Section

Materials

All chemicals were purchased from Sigma-Aldrich, Applichem, Merck, ABCR, Acros, Armar Chemicals, or Carl Roth and were of analytical grade or purer. Lysozyme from henn egg white (EC 3.2.17, ≥20000 U/g (pH 6.2)) was purchased from AppliChem and bovine serum albumin (BSA, ≥ 98 % content) from Roth. All chemicals were used without further purification.

Measurements

The turnover number (TON) and enantiomeric excess (ee) of the dihydroxylation and epoxidation of alkenes was determined by high performance liquid chromatography (HPLC) by using a HPLC system (LaChrom Elite, Hitachi) equipped with a chiral column (Chiralpack IC, Daicel Chemical Industries). As eluent, a mixture of n-heptane and isopropanol in a volume ratio of 9:1 was used. A flow rate of 0.5 mL min^{-1} and a diode array detector at 254 nm at 8 °C was applied. Calibration curves were performed with enantiopure samples of the respective diols and epoxides purchased from Sigma-Aldrich.

The DLS measurements were performed on a Malvern Zetasizer nano S at 20 °C. All samples were measured in toluene or chloroform. The equilibration time before measurement was 2 min.

All pH-values were determined using a Qph70 pH-meter (VWR International) calibrated by three point calibration (buffer solutions: pH 4.00/7.00/10.00 (HACH)).

Acylation of proteins (general procedure)

The protein modification with different acyl anhydrides was performed according to established literature protocols for N-acetylation.[18] First 0.3 µmol of the protein (4.2 mg for lysozyme, 19.9 mg for BSA) was dissolved in 3 mL of bidistilled water and cooled to 4 °C for the functionalization with acetic anhydride or propionic anhydride. Functionalization with hexanoic anhydride was carried out at room temperature because of the better suspension of hexanoic acid in water at this temperature. Afterwards the respective molar amount of anhydride for the desired degree of functionalization (see figure 2) was added and the mixture was allowed to react under vigorous stirring for 30 min at the preset temperature followed by subsequent lyophilisation to obtain a white solid. Then the solid was suspended in water and the pH was adjusted with NaOH and HCl solutions (0.01 M) prior to lyophilisation.

Determination of degree of acylation

The degree of functionalization of acetylated proteins was determined by an adjusted TNBS-assay according to literature known protocols.[20]. To 100 µL of protein mixture (1.4 mg mL^{-1} in water) were added 400 µL of a 0.1 M $NaHCO_3$ buffer at pH 8.5 and 250 µL of 0.01 % TNBS solution prepared in the same buffer. The solution was allowed to react at 40 °C for 2 h. Then, 250 µL 10 % (w/v) SDS was added followed by addition of 125 µL aqueous 1 M HCl. The absorbance (E) of the mixture was measured spectrophotometrically in a quartz glass cuvette (d = 1 cm) at 346 nm at 20 °C against a blank treated as above but with 500 µL of buffer instead of the protein solution. The number of free primary amino groups of the modified and unmodified protein were calculated according to Lambert-Beers law with a molar extinction coefficient of ε = 14600 mM^{-1} cm^{-1}.

$$free\ NH_2 = \frac{E \cdot V \cdot M_{protein}}{\varepsilon \cdot d \cdot m_{protein}}$$

Dihydroxylation of styrene (general procedure).

The acetylated protein (0.3 µmol) was dispersed in 10 mL of chloroform or toluene. Afterwards, 111 µL of $K_2OsO_2(OH)_4$ dissolved in methanol (1mg mL^{-1} in MeOH, 0.3 µmol for an MPR = 1) was given to the reaction mixture and is was stirred for 30 min. Then styrene (1mmol) was added. Finally, a 200 µL of a 5.5 M tBuOOH solution in decane (1100 µmol) was added and the reaction mixture was vigorously stirred at different temperatures (0 °C, 20 °C or 40 °C).

The reaction was stopped by filtration through a 0.2 µm PTFE-filter followed by the extraction with a 1 M aqueous sodium thiosulfate solution (2 x 10 mL). Afterwards, the phases were separated and the aqueous phase was extracted 5 times with 25 mL chloroform and the organic phases were combined. After removal of the solvent the obtained solid was characterized by HPLC.

Epoxidation of styrene with acetylated proteins as ligand (general procedure).

The acetylated protein (0.3 µmol) was dispersed in 5 mL of DCM. Afterwards, $RuCl_3$, $Ti(OMe)_4$ or $Mn(Ac)_2$ (1 mg/mL in MeOH, 0.3 µmol for an MPR = 1) was given to the reaction mixture and it was stirred for 30 min in a sealed vessel. After that the alkene (1 mmol) was added. Finally, a 5.5 M tBuOOH solution in decane (200 µl, 1100 µmol) was added and the reaction mixture was vigorously stirred at different temperatures (0 °C, 20 °C or 40 °C) in a sealed vessel.

The reaction was stopped by filtration through a 0.2 µm PTFE-filter followed by removal of the solvent. After that the obtained fluid was characterized by HPLC.

Keywords: artificial metalloenzymes • asymmetric dihydroxylation • asymmetric epoxidation • organic solvents • asymmetric catalysis

1. Illanes, A., *Enzyme Biocatalysis: principles and Applications*. 2008; p 392.
2. Bornscheuer, U. T.; Huisman, G. W.; Kazlauskas, R. J.; Lutz, S.; Moore, J. C.; Robins, K., Engineering the Third Wave of Biocatalysis. *Nature* **2012,** *485*, 185.
3. (a) Volkin, D. B.; Staubli, A.; Langer, R.; Klibanov, A. M., Enzyme thermoinactivation in anhydrous organic solvents. *Biotechnology and bioengineering* **1991,** *37* (9), 843-853; (b) Klibanov, A. M., Improving enzymes by using them in organic solvents. *Nature* **2001,** *409* (6817), 241-246.
4. (a) Luckarift, H. R.; Ku, B. S.; Dordick, J. S.; Spain, J. C., Silica-immobilized enzymes for multi-step synthesis in microfluidic devices. *Biotechnology and Bioengineering* **2007,** *98* (3), 701-705; (b) Tielmann, P.; Kierkels, H.; Zonta, A.; Ilie, A.; Reetz, M. T., Increasing the activity and enantioselectivity of lipases by sol-gel immobilization: further advancements of practical interest. *Nanoscale* **2014,** *6* (12), 6220-6228; (c) Mohamad, N. R.; Marzuki, N. H. C.; Buang, N. A.; Huyop, F.; Wahab, R. A., An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology & Biotechnological Equipment* **2015,** *29* (2), 205-220; (d) Matte, C. R.; Bussamara, R.; Dupont, J.; Rodrigues, R. C.; Hertz, P. F.; Ayub, M. A. Z., Immobilization of Thermomyces lanuginosus Lipase by Different Techniques on Immobead 150 Support: Characterization and Applications. *Applied Biochemistry and Biotechnology* **2014,** *172* (5), 2507-2520; (e) Sittko, I.; Kremser, K.; Roth, M.; Kuehne, S.; Stuhr, S.; Tiller, J. C., Amphiphilic polymer conetworks with defined nanostructure and tailored swelling behavior for exploring the activation of an entrapped lipase in organic solvents. *Polymer* **2015,** *64*, 122-129; (f) Dech, S.; Wruk, V.; Fik, C. P.; Tiller, J. C., Amphiphilic polymer conetworks derived from aqueous solutions for biocatalysis in organic solvents. *Polymer* **2012,** *53* (3), 701-707; (g) Plothe, R.; Sittko, I.; Lanfer, F.; Fortmann, M.; Roth, M.; Kolbach, V.; Tiller, J. C., Poly(2-ethyloxazoline) as matrix for highly active electrospun enzymes in organic solvents. *Biotechnology and Bioengineering* **2016**, n/a-n/a; (h) Savin, G.; Bruns, N.; Thomann, Y.; Tiller, J. C., Nanophase Separated Amphiphilic Microbeads. *Macromolecules* **2005,** *38* (18), 7536-7539.
5. Bruns, N.; Bannwarth, W.; Tiller, J. C., Amphiphilic conetworks as activating carriers for the enhancement of enzymatic activity in supercritical CO2. *Biotechnology and Bioengineering* **2008,** *101* (1), 19-26.
6. Schoenfeld, I.; Dech, S.; Ryabenky, B.; Daniel, B.; Glowacki, B.; Ladisch, R.; Tiller, J. C., Investigations on Diffusion Limitations of Biocatalyzed Reactions in Amphiphilic Polymer Conetworks in Organic Solvents. *Biotechnology and Bioengineering* **2013,** *110* (9), 2333-2342.
7. (a) Deuss, P. J.; den Heeten, R.; Laan, W.; Kamer, P. C. J., Bioinspired Catalyst Design and Artificial Metalloenzymes. *Chem. - Eur. J.* **2011,** *17*, 4680; (b) Yu, F. T.; Cangelosi, V. M.; Zastrow, M. L.; Tegoni, M.; Plegaria, J. S.; Tebo, A. G.; Mocny, C. S.; Ruckthong, L.; Qayyum, H.; Pecoraro, V. L., Protein Design: Toward Functional Metalloenzymes. *Chem. Rev.* **2014,** *114*, 3495; (c) Schwizer, F.; Okamoto, Y.; Heinisch, T.; Gu, Y.; Pellizzoni, M. M.; Lebrun, V.; Reuter, R.; Köhler, V.; Lewis, J. C.; Ward, T. R., Artificial Metalloenzymes: Reaction Scope and Optimization Strategies. *Chemical Reviews* **2017**; (d) Trindler, C.; Ward, T. R., Artificial Metalloenzymes. In *Effects of Nanoconfinement on Catalysis*, Poli, R., Ed. Springer International Publishing: Cham, 2017; pp 49-82; (e) Lu, Y.; Berry, S. M.; Pfister, T. D., Engineering Novel Metalloproteins: Design of Metal-Binding Sites into Native Protein Scaffolds. *Chem. Rev.* **2001,** *101*, 3047; (f) Pellizzoni, M. M.; Schwizer, F.; Wood, C. W.; Sabatino, V.; Cotelle, Y.; Matile, S.; Woolfson, D. N.; Ward, T. R., Chimeric Streptavidins as Host Proteins for Artificial Metalloenzymes. *ACS Catalysis* **2018,** *8* (2), 1476-1484; (g) Hestericová, M.; Heinisch, T.; Alonso-Cotchico, L.; Maréchal, J.-D.; Vidossich, P.; Ward, T. R., Directed Evolution of an Artificial Imine Reductase. *Angewandte Chemie International Edition* **2018,** *57* (7), 1863-1868; (h) Rebelein, J. G.; Ward, T. R., In vivo catalyzed new-to-nature reactions. *Current Opinion in Biotechnology* **2018,** *53*, 106-114; (i) Wieszczycka, K.; Staszak, K., Artificial metalloenzymes as catalysts in non-natural compounds synthesis. *Coordination Chemistry Reviews* **2017**; (j) Pàmies, O.; Diéguez, M.; Bäckvall, J.-E., Artificial Metalloenzymes in Asymmetric Catalysis: Key Developments and Future Directions. *Advanced Synthesis & Catalysis* **2015**, n/a-n/a; (k) Diéguez, M., Bäckvall, J.-E., Pàmies, O.(eds), *Artificial Metalloenzymes and MetalloDNAzymes in Catalysis: From Design to Applications*. Wiley-VCH: Weinheim, 2018.
8. Yamamura, K.; Kaiser, E. T., Studies on the Oxidase Activity of Copper(Ii) Carboxypeptidase A. *J. Chem. Soc., Chem. Commun.* **1976**, 830.
9. (a) Das, P. K.; Caaveiro, J. M. M.; Luque, S.; Klibanov, A. M., Asymmetric Sulfoxidations Mediated by Chymotrypsin. *Biotechnologie and bioengineering* **2002,** *78* (1), 104-109; (b) Esmieu, C.; Cherrier, M. V.; Amara, P.; Girgenti, E.; Marchi-Delapierre, C.; Oddon, F.; Iannello, M.; Jorge-Robin, A.; Cavazza, C.; Ménage, S., An Artificial Oxygenase Built from Scratch: Substrate Binding Site Identified Using a Docking Approach. *Angew. Chem., Int. Ed.* **2013,** *52*, 3922.
10. (a) Collot, J.; Gradinaru, J.; Humbert, N.; Skander, M.; Zocchi, A.; Ward, T. R., Artificial Metalloenzymes for Enantioselective Catalysis Based on Biotin-Avidin. *J. Am. Chem. Soc.* **2003,** *125*, 9030; (b) Reetz, M. T.; Peyralans, J. J. P.; Maichele, A.; Fu, Y.; Maywald, M., Directed Evolution of Hybrid Enzymes: Evolving Enantioselectivity of an Achiral Rh-Complex Anchored to a Protein. *Chem. Commun.* **2006**, 4318; (c) Wilson, M. E.; Whitesides, G. M., Conversion of a Protein to a Homogeneous Asymmetric Hydrogenation Catalyst by Site-Specific Modification with a Diphosphinerhodium(I) Moiety. *J. Am. Chem. Soc.* **1978,** *100*, 306.
11. (a) Podtetenieff, J.; Taglieber, A.; Bill, E.; Reijerse, E. J.; Reetz, M. T., An Artificial Metalloenzyme: Creation of a Designed Copper Binding Site in a Thermostable Protein. *Angew. Chem., Int. Ed.* **2010,** *49*, 5151; (b) Reetz, M. T.; Jiao, N., Copper–Phthalocyanine Conjugates of Serum Albumins as Enantioselective Catalysts in Diels–Alder Reactions. *Angew. Chem., Int. Ed.* **2006,** *45*, 2416.
12. (a) Quinto, T.; Haussinger, D.; Kohler, V.; Ward, T. R., Artificial metalloenzymes for the diastereoselective reduction of NAD+ to NAD2H. *Organic & Biomolecular Chemistry* **2015,** *13* (2), 357-360; (b) Quinto, T.; Koehler, V.; Ward, T. R., Recent Trends in Biomimetic Nadh Regeneration. *Top. Catal.* **2014,** *57*, 321.
13. (a) Koehler, V.; Mao, J.; Heinisch, T.; Pordea, A.; Sardo, A.; Wilson, Y. M.; Knoerr, L.; Creus, M.; Prost, J. C.; Schirmer, T., Oso4·Streptavidin: A Tunable Hybrid Catalyst for the Enantioselective Cis-Dihydroxylation of Olefins. *Angew. Chem., Int. Ed.* **2011,** *50*, 10863; (b) Kokubo, T.; Sugimoto, T.; Uchida, T.; Tanimoto, S.; Okano, M., The Bovine Serum Albumin-2-Phenylpropane-1,2-Diolatodioxo-Osmium(Vi) Complex as an Enantioselective Catalyst for Cis-Hydroxylation of Alkenes. *J. Chem. Soc., Chem. Commun.* **1983,** *0*, 769; (c) Jonsson, S. Y.; Färnegårdh, K.; Bäckvall, J.-E., Osmium-Catalyzed Asymmetric Dihydroxylation of Olefins by H2O2 Using a Biomimetic Flavin-Based Coupled Catalytic System. *Journal of the American Chemical Society* **2001,** *123* (7), 1365-1371; (d) Fujieda, N.; Nakano, T.; Taniguchi, Y.; Ichihashi, H.; Sugimoto, H.; Morimoto, Y.; Nishikawa, Y.; Kurisu, G.; Itoh, S., A Well-Defined Osmium–Cupin Complex: Hyperstable Artificial Osmium Peroxygenase. *Journal of the American Chemical Society* **2017,** *139* (14), 5149-5155.
14. (a) Fernández-Gacio, A.; Codina, A.; Fastrez, J.; Riant, O.; Soumillion, P., Transforming Carbonic Anhydrase into Epoxide Synthase by Metal Exchange. *ChemBioChem* **2006,** *7* (7), 1013-1016; (b) Allard, M.; Dupont, C.; Munoz Robles, V.; Doucet, N.; Lledos, A.; Marechal, J. D.; Urvoas, A.; Mahy, J. P.; Ricoux, R., Incorporation of Manganese Complexes into Xylanase: New Artificial

Metalloenzymes for Enantioselective Epoxidation. *ChemBioChem* **2012,** *13*, 240; (c) Okrasa, K.; Kazlauskas, R. J., Manganese-Substituted Carbonic Anhydrase as a New Peroxidase. *Chemistry – A European Journal* **2006,** *12* (6), 1587-1596.
15. (a) Thomas, C. M.; Ward, T. R., Artificial Metalloenzymes: Proteins as Hosts for Enantioselective Catalysis. *Chem. Soc. Rev.* **2005,** *34*, 337; (b) Colonna, S.; Manfredi, A.; Spadoni, M., The Effect of Organic Cosolvents on the Enantioselectivity in the Bovine Serum Albumin Catalyzed Weitz-Scheffer Condensation. *Tetrahedron Lett.* **1987,** *28*, 1577; (c) Laan, W.; Munoz, B. K.; den Heeten, R.; Kamer, P. C., Artificial Metalloenzymes through Cysteine-Selective Conjugation of Phosphines to Photoactive Yellow Protein. *ChemBioChem* **2010,** *11*, 1236.
16. (a) Konieczny, S.; Krumm, C.; Doert, D.; Neufeld, K.; Tiller, J. C., Investigations on the activity of poly(2-oxazoline) enzyme conjugates dissolved in organic solvents. *Journal of Biotechnology* **2014,** *181* (0), 55-63; (b) Konieczny, S.; Fik, C. P.; Averesch, N. J.; Tiller, J. C., Organosoluble Enzyme Conjugates with Poly(2-Oxazoline)S Via Pyromellitic Acid Dianhydride. *J. Biotechnol.* **2012,** *159*, 195; (c) Leurs, M.; Tiller, J. C., Chapter Seventeen - Nanoarmored Enzymes for Organic Enzymology: Synthesis and Characterization of Poly(2-Alkyloxazoline)–Enzyme Conjugates. In *Methods in Enzymology*, Kumar, C. V., Ed. Academic Press: 2017; Vol. 590, pp 413-444.
17. (a) Leurs, M.; Spiekermann, P. S.; Tiller, J. C., Optimization of and Mechanistic Considerations for the Enantioselective Dihydroxylation of Styrene Catalyzed by Osmate-Laccase-Poly(2-Methyloxazoline) in Organic Solvents. *ChemCatChem* **2016,** *8*, 593; (b) Konieczny, S.; Leurs, M.; Tiller, J. C., Polymer Enzyme Conjugates as Chiral Ligands for Sharpless Dihydroxylation of Alkenes in Organic Solvents. *ChemBioChem* **2015,** *16* (1), 83-90.
18. Tiller, J. C.; Bonner, G.; Pan, L.-C.; Klibanov, A. M., Improving biomaterial properties of collagen films by chemical modification. *Biotechnology and Bioengineering* **2001,** *73* (3), 246-252.
19. Tanford, C.; Wagner, M. L., Hydrogen Ion Equilibria of Lysozyme1,2. *Journal of the American Chemical Society* **1954,** *76* (12), 3331-3336.
20. (a) Habeeb, A. F. S. A., Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Analytical Biochemistry* **1966,** *14* (3), 328-336; (b) Sashidhar, R. B.; Capoor, A. K.; Ramana, D., Quantitation of ϵ-amino group using amino acids as reference standards by trinitrobenzene sulfonic acid: A simple spectrophotometric method for the estimation of hapten to carrier protein ratio. *Journal of Immunological Methods* **1994,** *167* (1), 121-127.
21. (a) Tse, M. K.; Döbler, C.; Bhor, S.; Klawonn, M.; Mägerlein, W.; Hugl, H.; Beller, M., Development of a Ruthenium-Catalyzed Asymmetric Epoxidation Procedure with Hydrogen Peroxide as the Oxidant. *Angewandte Chemie International Edition* **2004,** *43* (39), 5255-5260; (b) Balavoine, G.; Eskenazi, C.; Meunier, F.; Rivière, H., The stereospecific epoxidation of olefins catalysed by ruthenium. *Tetrahedron Letters* **1984,** *25* (30), 3187-3190; (c) Ferrer, Í.; Fontrodona, X.; Roig, A.; Rodríguez, M.; Romero, I., A Recoverable Ruthenium Aqua Complex Supported on Silica Particles: An Efficient Epoxidation Catalyst. *Chemistry – A European Journal* **2017,** *23* (17), 4096-4107; (d) Ujwaldev, S. M.; Sindhu, K. S.; Thankachan, A. P.; Anilkumar, G., Recent developments and perspectives in the ruthenium-catalyzed olefin epoxidation. *Tetrahedron* **2016,** *72* (41), 6175-6190; (e) McGarrigle, E. M.; Gilheany, D. G., Chromium−salen and Manganese−salen Promoted Epoxidation of Alkenes. *Chemical Reviews* **2005,** *105* (5), 1563-1602; (f) Sirijaraensre, J.; Limtrakul, J., Theoretical investigation on reaction pathways for ethylene epoxidation on Ti-decorated graphene. *Structural Chemistry* **2018,** *29* (1), 159-170; (g) Karjalainen, J.; Hormi, O.; Sherrington, D., Efficient Polymer-Supported Sharpless Alkene Epoxidation Catalyst. *Molecules* **1998,** *3* (3), 51.
22. (a) Danov, S. M.; Kazantsev, O. A.; Esipovich, A. L.; Belousov, A. S.; Rogozhin, A. E.; Kanakov, E. A., Recent advances in the field of selective epoxidation of vegetable oils and their derivatives: a review and perspective. *Catalysis Science & Technology* **2017,** *7* (17), 3659-3675; (b) de Boer, J. W.; Browne, W. R.; Brinksma, J.; Alsters, P. L.; Hage, R.; Feringa, B. L., Mechanism of Cis-Dihydroxylation and Epoxidation of Alkenes by Highly H2O2 Efficient Dinuclear Manganese Catalysts. *Inorganic Chemistry* **2007,** *46* (16), 6353-6372.
23. Shoichet, B. K.; Baase, W. A.; Kuroki, R.; Matthews, B. W., A relationship between protein stability and protein function. *Proceedings of the National Academy of Sciences* **1995,** *92* (2), 452-456.
24. Majorek, K. A.; Porebski, P. J.; Dayal, A.; Zimmerman, M. D.; Jablonska, K.; Stewart, A. J.; Chruszcz, M.; Minor, W., Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. *Molecular Immunology* **2012,** *52* (3), 174-182.
25. Corrado, M. L.; Knaus, T.; Mutti, F. G., A Chimeric Styrene Monooxygenase with Increased Efficiency in Asymmetric Biocatalytic Epoxidation. *ChemBioChem* **2018,** *19* (7), 679-686.
26. Hollmann, F.; Lin, P.-C.; Witholt, B.; Schmid, A., Stereospecific Biocatalytic Epoxidation: The First Example of Direct Regeneration of a FAD-Dependent Monooxygenase for Catalysis. *Journal of the American Chemical Society* **2003,** *125* (27), 8209-8217.

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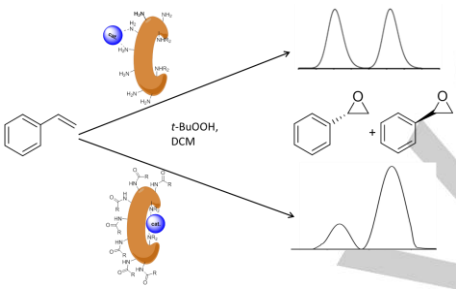
Entry for the Table of Contents

Layout 1:

FULL PAPER

The commercially available proteins lysozyme (LYS) and bovine serum albumin (BSA) were converted into highly active and enantioselective artificial metalloenzymes for dihydroxylation and epoxidation by acylation of the primary amino groups, which affords the proteins to take up metal salts in the core of the protein.



Melanie Leurs[a], Bjoern Dorn[a],Sascha Wilhelm[a] , Magiliny Manisegaran[a] and Joerg. C. Tiller[a]*

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Multicore Artificial Metalloenzymes derived from acylated proteins as catalysts for the enantioselective dihydroxylation and epoxidation of styrene derivatives.