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ARTICLE

Artificial metalloenzymes derived from bovine β-lactoglobulin for the asymmetric transfer hydrogenation of an aryl ketone – Synthesis, characterization and catalytic activity

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A series of diimines derived from saturated and unsaturated fatty acids and including a dipyridylamine (dpa) or a bispyridylmethane (bpm) scaffold as chelating moiety were synthesized and characterized spectroscopically. Complexation by $[LM(\mu\text{-}Cl)Cl]_2$ (M = Ru, L = *p*-cymene; M = Rh, L = Cp*) afforded the monocationic, mononuclear complexes of general formula [LM(N^N)Cl]Cl with N^N being the diimine ligand. Unsurprisingly, these new complexes catalysed the transfer hydrogenation of an activated aromatic ketone, namely 2,2,2-trifluoroacetophenone (TFACP), in water at neutral pH and mild temperature in the presence of formate as hydrogen donor. The catalytic activity of the complexes expressed as TOF was shown to depend not only on the metal (Ru or Rh) but also on the chelating entity (dpa or bpm) and the length and nature of the lipidic chain tethered to it. Incorporation of the complexes within bovine β-lactoglobulin (βLG) as protein host was studied by circular dichroism and fluorescence spectroscopy and again noticeable differences were observed between the saturated and unsaturated fatty acid derivatives. Eventually, the ability of the protein hybrids to catalyse the transfer hydrogenation of TFACP was demonstrated. Good-to-quantitative conversions in the corresponding alcohol were reached within 72 h with the rhodium (III) hybrids and the best enantioselectivities (up to 32% ee for the (R)-enantiomer) were measured with the Rh(III) cofactors derived from palmitic and stearic acids once incorporated in the isoform A of βLG.

Introduction

The design of artificial metalloproteins by assembling of metal / metal complexes to biomolecular scaffolds is the subject of intense research.[1] The rationale is either to reproduce the structure and function of native metalloproteins (biomimetic approach)[2] or to confer new functionalities to biopolymers by taking advantage of the known reactivity of synthetic metal complexes.[3] Such an endeavour is likely to meet success thanks to the intimate knowledge of both the protein scaffold that hosts the metal entity and the interaction(s) between the metal and its chelating ligands (first sphere of coordination) and the protein environment (second sphere of coordination). To rationally design an artificial metalloenzyme, that is, to place a metal center within a protein host in a controlled fashion, two strategies are available: the non-covalent strategy including dative and supramolecular interactions and the covalent anchoring strategy.[4] These strategies have their pros and cons but so far the supramolecular anchoring approach using the biotin / (strept)avidin association has appeared the most ubiquitous and proved the most efficient in terms of reactivity / selectivity.[5]

Asymmetric transfer hydrogenation (ATH) of ketones is an attractive route to synthesize chiral secondary alcohols. This reaction is generally catalysed by Ru(II), Rh(III) or Ir(III) complexes of chiral diamine ligands in the presence of hydrogen donors.[6] Further studies have shown that this reaction can also take place in aqueous medium using formate as hydrogen source.[7, 8] Interestingly, achiral diimine ligands such as bipyridine,[9, 10] phenanthroline,[11] bipyrimidine[12] and 2,2'-dipyridylamine (dpa)[13] can also catalyse transfer hydrogenation of ketones in water with formate, yielding the secondary alcohols in racemic form.

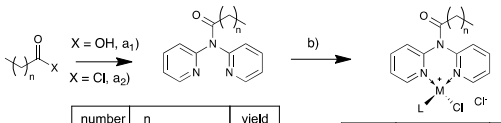
Artificial transfer hydrogenases have been successfully built up by assembling of biotinylated d^6-piano stool complexes of Ru, Rh or Ir to (strept)avidin. These hybrid species catalyse the reduction of aryl ketones with high yield and selectivity.[14] Our

own efforts towards the design of artificial metalloenzymes first focused on papain as protein host. This scaffold was chosen because of the presence of a reactive thiol function located within a well-defined wide cavity and amenable to selective chemical modification.[15] Several metal complexes were successfully anchored to papain, which was subsequently conferred with novel catalytic activities.[16] Unfortunately very limited enantioselectivites were so far observed in the transfer hydrogenation of 2,2,2-trifluoroacetophenone (TFACP) with metallopapains, probably owing to the surface location of the metal active centre.[17] In search for an alternative protein host, we set our choice on bovine β-lactoglobulin (βLG). βLG is the most abundant whey protein of cow's milk. Its sequence contains 162 amino acids, two of them differing in the two major genetic variants A and B found in cow's milk. Most importantly, βLG displays a typical 8-stranded, antiparallel β-barrel folding, forming a calyx lined with hydrophobic residues.[18] Although its exact function is not yet fully understood, its structural analogy with retinol-binding protein provides a good evidence that βLG is involved in the transport and uptake of vitamin A in suckling animals.[19] Besides retinol, βLG also binds other hydrophobic ligands such as fatty acids with submicromolar dissociation constants and close to 1:1 stoichiometry.[20] Structural studies showed that the fatty acid aliphatic tail always occupies the βLG hydrophobic cavity while the carboxyl group points towards the calyx entrance.[21-23] Such a configuration is reminiscent of the biotin / (strept)avidin association. By analogy, a metal chelate may be introduced at the carboxylic acid function of fatty acids, which would position the metal centre at the entrance of the βLG calyx. Consequently we undertook the synthesis of fatty acid-derived diimine ligands and their d^6-piano stool M complexes (M = Ru or Rh) and tested their ability to catalyse the transfer hydrogenation of TFACP. Next, we studied the association of the metal complexes with βLG by circular dichroism and fluorescence spectroscopy. Finally, the ability of the hybrid species to catalyse the ATH of TFACP was demonstrated with the most effective systems relying on saturated fatty acid-derived metal cofactors. Some of the results have been published as a preliminary communication.[24]

Results and discussion

Synthesis of fatty acid-derived metal cofactors

A first series of diimine ligands **1 – 7** derived from saturated and unsaturated fatty acids and carrying the dpa chelate motif was obtained by acylation of commercially available 2,2'-dipyridylamine by the acid chlorides of capric (C10:0), lauric (C12:0), myristic (14:0), palmitic (16:0), stearic (18:0),[25] oleic (18:1 Δ^9) and linoleic (18:2, $\Delta^{9,12}$) in 44 to 97% yield (Scheme 1).

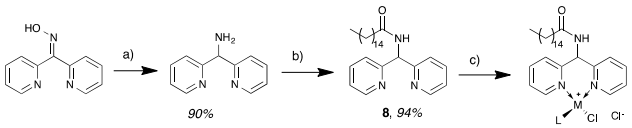


number	n	yield
1	8	*78%*
2	10	*44%*
3	12	*97%*
4	14	*73%*
5	16	*97%*
6	16, *trans*-Δ^9	*83%*
7	16, *trans*-$\Delta^{9,12}$	*69%*

number	n	M	L	yield
1-Rh	8	Rh	Cp*	*75%*
2-Rh	10	Rh	Cp*	*89%*
2-Ru	10	Ru	*p*-cym	*96%*
3-Rh	12	Rh	Cp*	*66%*
4-Rh	14	Rh	Cp*	*85%*
4-Ru	14	Ru	*p*-cym	*85%*
5-Rh	16	Rh	Cp*	*73%*
6-Rh	16, *trans*-Δ^9	Rh	Cp*	*83%*
7-Rh	16, *trans*-$\Delta^{9,12}$	Rh	Cp*	*76%*

Scheme 1 a_1) i) $(COCl)_2$, toluene, 2 h, ii) 2,2'-dipyridylamine, THF, 1 h, iii) TEA, 2 days; a_2) 2,2'-dipyridylamine, TEA, DCM, 18 h; b) $[LM(\mu\text{-}Cl)Cl]_2$, DCM, 16 h

An additional diimine ligand **8,** including a bispyridylmethane (bpm) chelate, was prepared in 2 steps using the commercially available oxime as starting material (Scheme 2). Di(2-pyridyl)methylamine was first obtained according to a literature procedure[26] and was further acylated with palmitoyl chloride to yield the secondary amide in nearly quantitative yield.



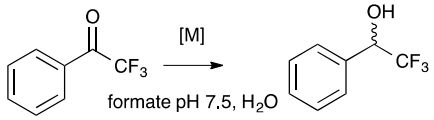
number	M	L	yield
8-Rh	Rh	Cp*	80%
8-Ru	Ru	*p*-cym	80%

Scheme 2 a) NH_3, Zn, NH_4OAc, $EtOH/H_2O$, Δ; b) palmitoyl chloride, TEA, DCM, 18 h; c) $[LM(\mu\text{-}Cl)Cl]_2$, DCM, 16 h

Reaction of the diimines with 0.5 eq. $[(\eta^6\text{-}p\text{-cymene})Ru(\mu\text{-}Cl)Cl]_2$ or $[(\eta^5\text{-}Cp^*)Rh(\mu\text{-}Cl)Cl]_2$ in DCM afforded the monocationic, mononuclear d^6-piano stool Ru(II) or Rh(III) complexes in high to quantitative yield whose structure was confirmed by spectroscopic analyses.

Aqueous transfer hydrogenation of trifluoroacetophenone by metal complexes

TFACP was chosen as benchmark substrate to delineate the catalytic activity of the newly synthesized complexes in aqueous transfer hydrogenation (TH) reaction using formate as hydrogen source (Scheme 3).



Scheme 3 (Asymmetric) transfer hydrogenation of TFACP

This substrate can be readily reduced thanks to the electron-attracting character of the CF_3 substituent.[10, 17, 27] Reactions were carried out at the analytical scale in experimental conditions compatible with βLG. Substrate and product were identified and quantified by reverse-phase HPLC by comparison with authentic compounds. Reaction times and conversions in α-(trifluoromethyl)benzyl alcohol are gathered in Table 1. The following conclusions can be drawn from this

set of experiments. First, at neutral pH and moderate temperature, the Rh(III) complexes were always much more active than their Ru(II) counterparts. For instance, quantitative conversion was reached within ca. 2 h with **2-Rh** (entry 2) while it barely reached 33% after 72 h with the Ru(II) analogue **2-Ru** (entry 3). Similarly, conversion of TFACP reached 91% after 7.5 h in the presence of **8-Rh** (entry 10) while only 1% TFACP was converted after 72 h with the Ru(II) counterpart **8-Ru** (entry 11). Second, the dpa-based Rh(III) complex **4-Rh** derived from palmitic acid was more active than the bpm-based analogue **8-Rh** (entries 5 and 10). More subtle differences were also noticed within the dpa-Rh(III) series, depending on the nature of the fatty acid side chain (see below). On the whole, the most reactive complex was **7-Rh** for which total conversion was reached within 75 min (entry 9).

Table 1 Transfer hydrogenation of TFACP in the presence of complexes[a]

Entry	Catalyst	Conversion, % (time, h)	TOF, h^{-1} [b]
1	**1-Rh**	Quant. (1.9)	32
2	**2-Rh**	Quant. (2.25)	27
3	**2-Ru**	33 (72)	0.1
4	**3-Rh**	Quant. (2.3)	20
5	**4-Rh**	Quant. (4.7)	6
6	**4-Ru**	51 (48)	0.7
7	**5-Rh**	96 (12)	4
8	**6-Rh**	Quant. (3.25)	16
9	**7-Rh**	Quant. (1.25)	69
10	**8-Rh**	91 (7.5)	14
11	**8-Ru**	1 (72)	-

[a] [S] = 10 mM, [cat] = 0.2 mM, [formate] = 1 M pH 7.4, 40°C (v=1 mL); conversion measured by RP-HPLC; [b] TOF (turnover frequency) defined as mol product / mol catalyst / time

Kinetics of transfer hydrogenation of TFACP was monitored in more detail and the turnover frequencies (TOF's) determined at early reaction time. Significant differences of reactivity were noticed between the complexes under study (Table 1). For the Rh(III) complexes derived for saturated fatty acids, TOF's spanned over a large range of values, i.e. from 4 h^{-1} for the C18 alkyl chain derivative **5-Rh** (entry 7) to 32 h^{-1} for the C10 alkyl chain **1-Rh** (entry 1). Although the first sphere of coordination around the metal centre was the same, the catalytic activity of the metal complexes was inversely related to the length of the fatty acid tail. Incidentally, complex **4-Rh** gave a TOF twice lower than the homologous complex **8-Rh** (entries 5 and 10). Kinetic plots of TH of TFACP catalysed by the palmitic acid derived Rh(III) catalysts **4-Rh** and **8-Rh** are shown in Fig. 1 to illustrate this point. Immediate appearance of alcohol product was witnessed with **8-Rh** whereas a lag period of 90 min was observed on the kinetic curve for **4-Rh**. Consequently a slight modification of the diimine ligand structure had a marked influence of the rate of TH but despite the lower TOF of **4-Rh** compared to **8-Rh**, full conversion of TFACP was reached in a shorter time within the former catalyst.

As mentioned above, the dpa-Ru(II) complexes afforded much slower kinetics of hydrogenation than their Rh(III) counterparts (Table 1, entries 3 and 6). We also found that the presence of unsaturations on the fatty acid chain greatly increased the rate of TH of TFACP (entries 7 – 9) with the linoleic acid derivative being the most active catalyst by far.

A second catalytic run in the presence of 4-Rh gave a TOF of 2.4 h^{-1} (instead of 6, entry 5), indicating that the complex was still able to catalyse the TF of TFACP. The lower rate may be due to a lower concentration of formate in the medium at the second run, which leads in turn to a reduced reaction rate (see below).

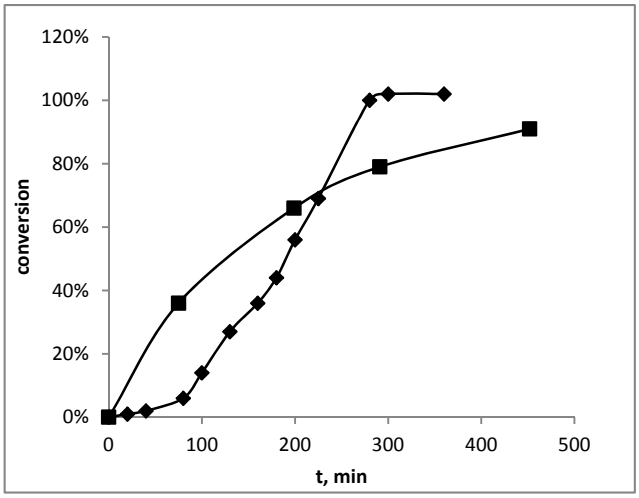


Fig. 1 Graph of conversion versus time for the aqueous TH of TFACP in the presence of **8-Rh** (◆) or **4-Rh** (♦). Conditions: [S] = 10 mM, [cat] = 0.2 mM, [formate] = 1 M pH 7.5, 40°C (v=1 mL)

Conversion of TFACP catalysed by **2-Rh** along with evolution of pH as a function of time is shown in Fig. 2. Not surprisingly, the solution pH increased steadily during reaction as a consequence of formate consumption and decomposition into CO_2 and H_2[8] and paralleled the conversion evolution.

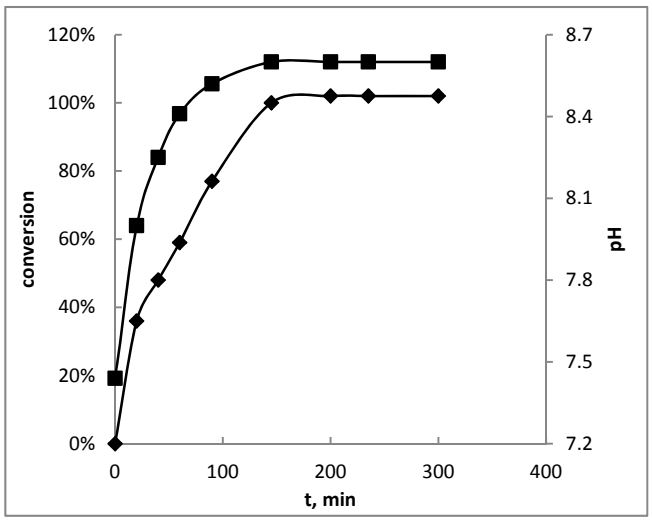


Fig. 2 Graph of conversion (♦) and pH (◆) versus time for the aqueous TH of TFACP in the presence of **2-Rh**. Conditions: [S] = 10 mM, [cat] = 0.2 mM, [formate] = 1 M pH 7.4, 40°C (v=1 mL)

Next the dependence of formate concentration on the rate of TH of TFACP was studied for **2-Rh** and **4-Rh** (ESI, Fig. S1). It

was observed that the rate of reaction increased almost proportionally with the concentration of formate in the range between 0.5 (F/S = 50) and 1 M (F/S = 100). Again a large difference of reactivity was noticed between **2-Rh** and **4-Rh**.

The influence of the initial solution pH on the conversion of TFACP was investigated for **2-Ru** and **2-Rh** (Fig. S2). The pH was varied in the range between 2 and 7 by adjusting the proportion between formic acid and sodium formate in the reaction mixtures. A slight pH dependency was observed for **2-Rh** after 1 h reaction but high to complete conversions were measured in the whole range of pH after 25 h. Reactivity of **2-Ru** was again very sluggish as compared to **2-Rh**.

The reaction scope was lastly investigated on a series of closely related aryl ketones, namely acetophenone, 4-methylacetophenone, 4-bromoacetophenone and 2-methoxyacetophenone. Under the experimental conditions used for TFACP, no alcohol was observed after 18 h in the presence of 2 mol% **2-Rh** for any of the substrates tested. Under harsher conditions (2 M formate and 70°C), very low amounts of reduction products were detected by GC except for aceto-phenone for which conversion was nil (Table 2). This indicates that the electron-attracting substituent CF_3 was required to activate the carbonyl function and favour its reduction.

Table 2 Aqueous transfer hydrogenation of several aryl ketones catalysed by **2-Rh**[a]

Substrate	Conversion, %
Acetophenone	0
4-methylacetophenone	2
4-bromoacetophenone	8
2-methoxyacetophenone	5

[a] [S] = 10 mM, [**2-Rh**] = 0.2 mM, [formate] = 1 M, pH 7.4, 70°C, 18h, GC analysis

Supramolecular assembling of βLG hybrids

Near-UV circular dichroism spectroscopy was employed to assess the incorporation of the ligands and the complexes within βLG. In a typical experiment, small aliquots of ligand / complex solution in EtOH or DMSO were added to a solution of βLG in phosphate buffer pH 7.5 in the range between 0.3:1 to 1.2:1 mole ratio of ligand / complex to protein. The near-uv CD spectrum was measured after each new addition and compared to the spectrum of βLG alone. As an example, Figure 3 depicts the evolution of the CD spectrum of mixtures of βLG and increasing amounts of **3-Rh**. The CD spectrum of βLG displays a pattern of negative bands between 250 and 300 nm owing to its aromatic residues.[28] Upon addition of **3-Rh**, the near uv CD spectrum underwent a noticeable change with a positive band gradually appearing at ca. 270 nm that overlaps the previous protein bands together with a broad negative band around 350 nm. These two bands are also observed on the absorption spectrum of **3-Rh** and assigned to ligand-centred and metal-to-ligand charge transfer transitions, respectively.[29] Thus, the presence of βLG undoubtedly placed **3-Rh** in a chiral environment inducing CD signals. Nearly no change of the spectrum was further observed between 0.9 and 1.2 eq. of **3-Rh** indicating that **3-Rh** bound to βLG with a 1:1 molar ratio.

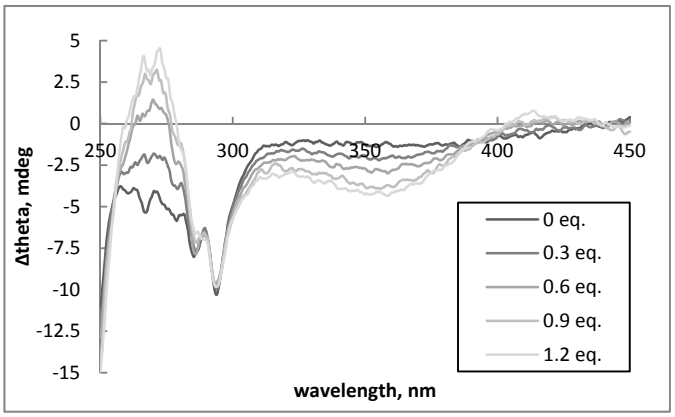


Fig. 3 Evolution of the near uv CD spectrum of βLG upon addition of increasing amounts of **3-Rh**. [βLG] = 59 μM in 20 mM phosphate pH 7.5

CD data for the other ligands / complexes are summarized in Table 3.

Table 3 Analysis of association of ligands / complexes to βLG by near uv CD spectroscopy

Ligand / complex	Extrinsic CD signal, λ of CD detection, nm
4	+, 271
4-Ru	-[a]
4-Rh	+, 350 and 271
8-Rh	none
8-Ru	None
2	+, 271
2-Ru	+, 270
2-Rh	+, 350 and 271
7-Rh	+, 350
3-Rh	+, 275 and 350
5-Rh	+, 275 and 350
6-Rh	None
1-Rh	none

[a] association detected upon competition with **4**

Upon addition of the dipyridylamide ligands derived from palmitic and lauric acid **4** and **2**, an induced positive signal centered at 271 nm was observed for, i.e. at the same position where the chromophore displays an absorption maximum. This band disappeared when excess palmitic acid was added to the mixture of βLG and **4** (Fig. S3). This band also progressively decreased when the solution containing βLG and **4** was gradually acidified, as a consequence of the dissociation of the βLG-**4** complex (Fig. S4). Such a behaviour was previously observed for the complexation of trans-parinaric acid to βLG[30] and was ascribed to the well-known pH-dependent conformational change called the Tanford transition[31] that was later on identified by structural analysis as a movement of the EF loop located at the open end of the central calyx and behaving as a lid at acidic pH.[32] Closing of the lid at low pH precludes ligands access to the calyx thus their association to the protein. These features put together give a good indication that **4** binds to the central cavity of βLG as palmitic acid does.[23]

Some of the Rh(III) complexes derived from the dipyridylamide ligands were also shown to induce CD signals upon complexation to βLG with bands at ca. 270 nm and 350 nm. Conversely, no change of the CD spectrum of βLG was observed in the presence of **4-Ru**. Thus a competition experiment was performed where **4-Ru** was gradually added to a mixture of βLG and 0.7 eq. **4**. The induced CD signal at 270 nm disappeared accordingly, indicating that **4-Ru** was able to displace the binding of **4** to βLG (Fig. S5). No such feature was found for the two complexes including the bispyridylmethane ligand **8-Ru** and **8-Rh** for which no change of the near-uv CD spectrum was observed either directly or indirectly. No extrinsic CD signal was also observed for the capric acid derivative **1-Rh** and for the unsaturated fatty acid derivative **6-Rh**. ESI –MS[33] and fluorescence spectroscopy[34] studies both showed that βLG displays low affinity for capric acid. Conversely, the absence of an extrinsic CD signal for **6-Rh** was rather unexpected since the parent oleic acid binds to βLG with high affinity ($K_d = 1 \pm 0.35$ μM).[21]

Complexation of non fluorescent saturated and unsaturated fatty acids together with other ligands to βLG can also be monitored by fluorescence spectroscopy of its two tryptophan residues Trp19 and Trp61.[20, 35] The former is located at the bottom of the calyx while the latter is located on an external loop and accessible to solvent. Comparative studies of bovine and equine βLG which lacks Trp61 have shown that Trp19, being in a hydrophobic environment, contributes for more than 70% of the total emission.[36] Accordingly, mutation of Trp19 by Ala induced a decrease of the intrinsic fluorescence of βLG by 80%.[37] We assumed we could apply fluorescence titration to investigate the assembling of the metal complexes to βLG and possibly determine the binding constant. A solution of βLG in phosphate buffer pH 7.5 was titrated by adding small increments of **2-Rh** or **4-Rh**. A control experiment was carried out with N-acetyl-L-tryptophanamide (NALTA) instead of βLG to subtract the inner filter effect due to reabsorption of the fluorophores emission by the complexes (Fig. S6).[38] Conversely to palmitic acid and the other fatty acids that induced an increase of fluorescence,[20] addition of **2-Rh** or **4-Rh** to βLG led to a strong quenching of the protein's intrinsic fluorescence, i.e. up to 80% (Fig. 4). The apparent dissociation constants K'_d and binding ratios n (Table 4) were deduced from the fluorescence data according to Cogan et al (Fig. S7).[38] **2-Rh** and **4-Rh** appeared to bind to βLG with dissociation constants in the same order of magnitude as those previously measured for the corresponding lipids. Hence, the presence of the bulky organometallic head tethered to the lipid tail did not entail binding of the complexes to the protein.

According to the literature, quenching of βLG fluorescence can be due to resonant energy transfer (RET) between excited Trp side chains and the complex, change of polarity in their neighbourhood or both effects.[35] But since there is a partial overlap between the fluorescence emission spectrum of βLG and the absorption spectra of **4-Rh** and **2-Rh** (Fig. S8), quenching by RET is likely to occur with the complexes acting as acceptors.

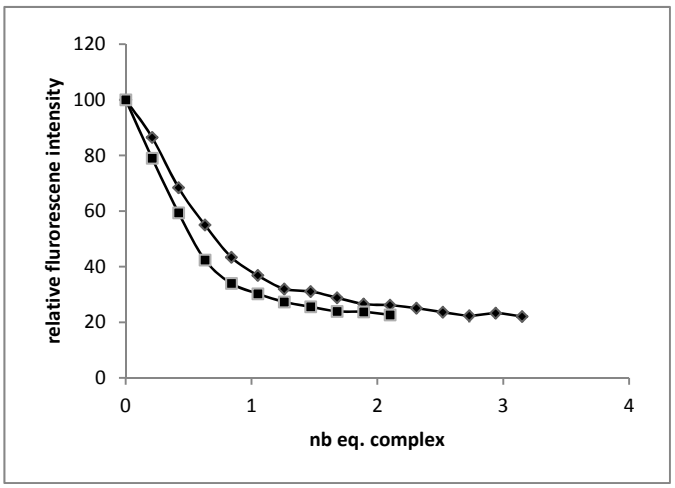


Fig. 4 Corrected fluorescence titration curves of βLG (2 μM in 20 mM phosphate pH 7.5) with **2-Rh** (♦) and **4-Rh** (◆). λ_{ex} = 290nm, t= 20°C

Table 4 Apparent dissociation constants (K'd) and apparent molar ratios (n) of **4-Rh**⊂βLG and **2-Rh**⊂βLG measured by fluorescence titration

Complex	K'd, μM	n	Ref.
4-Rh	0.28 ± 0.04	0.75 ± 0.01	This work
2-Rh	0.66 ± 0.05	0.93 ± 0.02	This work
Palmitic acid (C16:0)	0.1 ± 0.005	0.93 ± 0.11	[20]
Lauric acid (C12:0)	0.7 ± 0.16	0.86 ± 0.05	[20]

In summary, CD together with fluorescence spectroscopy provided solid clues as to the association of nearly all the complexes to βLG. X-ray structural analysis of the **2-Rh**⊂βLG(A) hybrid confirmed that the complex bound to the protein in a similar fashion as the parent lipid lauric acid and that the organometallic "head" was located at the entrance of the calyx where it adopted several conformations.[39]

Transfer hydrogenation of TFACP in the presence of metalloproteins

Finally, the ability of the βLG hybrids to catalyse the asymmetric transfer hydrogenation of TFACP was tested under the standard conditions previously set up for the complexes alone. To make sure that all the molecules of complex were indeed bound to the protein, the mole ratio of complex to protein was set to 0.7 and the pH set to 7.5. TOF's were measured at short conversion times (7 or 24 h) and are gathered in Table 5. The first point to be noticed is that all the hybrids catalysed the ATH of TFACP but at lower rates than the complexes alone. Second, except for the short alkyl chain derivative **1-Rh** and unlike what had been previously witnessed with the isolated complexes, TOF's were found relatively independent of the alkyl chain length and the presence of unsaturations. For instance, the rate of reaction catalysed by **5-Rh** was reduced by a factor of 3 in the presence of βLG while it was reduced by a factor of 50 for **7-Rh**. The behaviour of **2-Ru** was quite anomalous since the TOF was actually higher in the presence of βLG.

Conversion of TFACP and enantiomeric excess (ee) were measured by chiral HPLC after 48 or 72 h (Table 5). Conversions ranged from 15% to quantitative, while enantiomeric excesses (ee's) ranged from 7% to 32% in favour of the (*R*)-enantiomer. The complexes derived from the unsaturated lipids and that derived from capric acid gave high conversions but lower ee's than those derived from saturated lipids in the presence of βLG. Simultaneous high conversion and low ee may be explained by a lack of association between βLG and metal complex and / or partial dissociation of the complex from the protein host. Most of the time, low ee's correlated with absence of extrinsic CD signal except for **8-Rh** for which an ee of 16% was measured, indicating that **8-Rh** did associate with βLG. Hybrids of complexes derived from saturated lipids (other than capric acid) gave similar results in terms of both activity and selectivity.

The pure variants A and B taken separately yielded identical but lower conversions than the mixture of both isoforms (compare entries 3 and 4 to entry 2 or entries 12 and 13 to entry 11). Simultaneously, the enantioselectivity was significantly higher with the pure isoforms and identical for both pure isoforms. This difference of reactivity / selectivity between the mixture of isoforms and the pure variants is related to the fact that the mixture of isoforms is partially (and heterogeneously) lactosylated whereas the pure isoforms are not, according to ESI-MS analysis of the protein samples. Lactosylation arises from milk processing[40] and has been shown to induce a change of protein conformation possibly responsible for the difference of selectivity noticed above.[41]

Table 5 Transfer hydrogenation of TFACP in the presence of βLG hybrids[a]

entry	βLG	Cofactor	TOF, h^{-1}	Conversion, % (time, h)	ee, %
1	A+B	**1-Rh**	3.4	100 (72)	7
2	A+B	**2-Rh**	0.8	50 (48)	17
3	A	**2-Rh**	1.1	68 (72)	26
4	B	**2-Rh**	1.4	60 (48)	27
5	A+B	**2-Ru**	0.2	15 (72)	17
6	A+B	**3-Rh**	1.1	87 (72)	17
7	A	**3-Rh**	0.6	64 (72)	21
8	A+B	**4-Rh**	0.6	50 (48)	16
9	B	**4-Rh**	0.8	61 (72)	26
10[b]	A+B	**8-Rh**	0.2	29 (72)	16
11	A+B	**5-Rh**	1.4	88 (72)	16
12	A	**5-Rh**	0.9	59 (72)	32
13	B	**5-Rh**	1.1	56 (72)	30
14	A+B	**6-Rh**	1.9	99 (72)	12
15	A+B	**7-Rh**	1.4	94 (72)	15
16	A	**7-Rh**	1.8	92 (72)	12

[a] [S] = 5 mM, [cofactor] = 0.2 mM, [βLG] = 0.28 mM, [formate] = 1 M pH 7.5, 40°C (v=1 mL); conversion, TOF and ee measured by chiral HPLC; (R)-enantiomer was obtained in excess; [b] [S] = 1 mM, [cofactor] = 0.1 mM, [βLG] = 0.14 mM

Complementary experiments were carried out with **2-Rh**⊂βLG(B) in order to assess the influence of pH, buffer and formate concentration on the rate, conversion and ee (Table 6, Fig. S9). Since the formation of protein hybrid occurs at pH over 7.5, a limited range of pH was tested. By raising the initial pH to 8.7 or decreasing the concentration of formate to 0.5 M, the initial rate decreased by a factor of 2 while conversions at 72h were conserved. The ee's measured at 72h were also close, but for the catalytic run performed at pH 8.7, the ee significantly decreased with time, which might result from a slow dissociation of the cofactor from the protein host. Finally, addition of HEPES buffer to maintain the pH at 7.5 was detrimental to rate, conversion and ee.

Table 6 Transfer hydrogenation of TFACP in the presence of **2-Rh**⊂βLG(B) hybrid under various reaction conditions [a]

pH	[formate], M	TOF, h^{-1}	Conversion, %	ee, %
7.5	1	1.4	72	24
7.5	0.5	0.6	65	21
8.7	1	0.7	66	19
7.5[b]	1	0.2	52	15

[a] [S] = 5 mM, [**2-Rh**] = 0.1 mM, [βLG(B)] = 0.14 mM, 40°C, 72 h; [b] in the presence of 25 mM HEPES pH 7.5

Control experiments carried with **2-Rh** and lysozyme or avidin instead of βLG gave full conversion of TFACP within 72 h (TOF = 8 and 4 h^{-1}, respectively) but no significant ee. Another control experiment carried out with βLG, **4-Rh** and 2 mole eq. palmitic acid also afforded nearly complete conversion of TFACP (TOF = 2.2 h^{-1}) but no significant ee was measured. We interpret this result as the displacement of **4-Rh** from βLG's binding site by competitive binding of palmitic acid. Incidentally, the mere presence of a protein in the reaction medium induced a strong deceleration of the hydrogenation since the TOF measured with **2-Rh** is equal to 27 h^{-1} (see table 1, entry 2). This deceleration was previously assigned to competitive chelation by the protein.[27]

Conclusions

In summary, a large set of diimine ligands carrying various fatty acid substituents and their d^6-piano stool Ru / Rh complexes were synthesized and characterized spectroscopically. They were shown to catalyse the transfer hydrogenation of an activated aryl ketone in neat water using formate as hydrogen source with variable efficiency depending on the metal, fatty acid length and structure and formate concentration. CD and fluorescence spectroscopy were implemented to assess the association of the complexes to βLG. In the presence of βLG, all the metal complexes retained their ability to catalyse the ATH of TFACP while some of them gave preferentially the (*R*)-enantiomer of the corresponding alcohol with ee up to 32%. The crystal structure of one of the protein – complex ensembles now being available, we can contemplate the genetic optimization of the protein scaffold so as to eventually improve the metalloenzyme selectivity, with residues L39 and A83 being the most relevant targets for mutation.

Experimental

Transfer hydrogenation of TFACP catalysed by the complexes

Sodium formate (68.1 mg, final concentration: 1 M) was added to a solution of TFACP (10 mM in water, 1 mL) and the pH was adjusted to 7.5 with 0.1 M NaOH. A solution of complex (10 mM in DMSO, 20 µL, 0.02 eq.) was added with a micropipette and the mixture was stirred for up to 72 h at 40°C. Aliquots (10 µL) of the reaction mixture were taken periodically, diluted in 90 µL water and analysed by RP-HPLC. The pH was measured at the end of the reaction and was about 8.6.

Transfer hydrogenation of other aryl ketones catalysed by 2-Rh

Sodium formate (136 mg, final concentration: 2 M) was added to a solution of aryl ketone (10 mM in water, 1 mL) and the pH was adjusted to 7.5 with 0.1 M NaOH. A solution of complex (10 mM in DMSO, 20 µL, 0.02 eq.) was added and the mixture was stirred for 18 h at 70°C. After cooling to room temp., the aqueous solution was extracted with diisopropylether (900 µL) and the organic phase analysed by GC.

Transfer hydrogenation of TFACP catalysed by the metalloproteins

βLG (A, B or mixture, 2.7 mg, 0.028 equiv.) was added to a solution of Na formate in water (1 M, pH adjusted to 7.5 with 0.1 M NaOH, 1 mL) and the mixture was stirred for 1 min. A solution of complex (10 mM in DMSO, 10 µl, 0.02 equiv.) was added and the reaction mixture was stirred for 1 min at room temperature. Finally, TFACP (0.7 µL, final concentration 5 mM) was added and the mixture was stirred for up to 72 h at 40°C. Aliquots (10 µL) of the reaction mixture were taken periodically, diluted in 90 µL water and submitted to chiral HPLC analysis.

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Electronic Supplementary Information (ESI) available: [synthetical procedures, ^{1}H and ^{13}C NMR spectra, CD spectra, fluorescence titrations, GC and HPLC analyses]. See DOI: 10.1039/b000000x/

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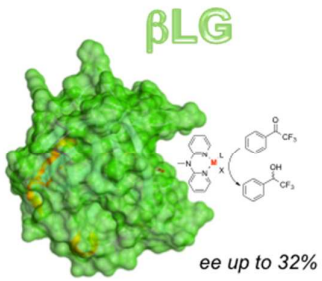

Protein hybrids resulting from the supramolecular anchoring to bovine β-lactoglobulin of fatty acid-derived Rh(III) diimine complexes catalysed the asymmetric transfer hydrogenation of trifluoroacetophenone with up to 32% ee.