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On the nature of mutual inactivation between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and enzymes – analysis and potential remedies

Maël Poizat, Isabel W.C.E. Arends, Frank Hollmann *

Delft University of Technology, Department of Biotechnology, Biocatalysis and Organic Chemistry Group, Julianalaan 136, 2628 BL Delft, The Netherlands

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ABSTRACT

Pentamethylcyclopentadienyl rhodium bipyridine ($[Cp^*Rh(bpy)(H_2O)]^{2+}$) is a versatile catalyst to promote biocatalytic redox reactions. However, its major drawback lies in the mutual inactivation of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and the biocatalyst. This interaction was investigated using the alcohol dehydrogenase from *Thermus* sp. ATN1 (TADH) as model enzyme. TADH binds 4 equiv. of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ without detectable decrease in catalytic activity and stability. Higher molar ratios lead to time-, temperature-, and concentration-dependent inactivation of the enzyme suggesting $[Cp^*Rh(bpy)(H_2O)]^{2+}$ to function as an 'unfolding catalyst'. This detrimental activity can be circumvented using strongly coordinating buffers (e.g. $(NH_4)_2SO_4$) while preserving its activity as NAD(P)H regeneration catalyst under electrochemical reaction conditions.

1. Introduction

NAD(P)-dependent oxidoreductases are valuable tools for the synthesis of chiral compounds. Especially, the enantiospecific reduction of prochiral ketones has attracted considerable academic [1,2] and industrial [3,4] interest. Due to the high cost of the nicotinamide cofactors (NAD(P)), *in situ* cofactor regeneration is required for preparative applications. Besides a wealth of enzymatic cofactor regeneration approaches [5–10], a range of non-enzymatic approaches have been proposed [11,12], with the organometallic pentamethylcyclopentadienyl rhodium bipyridine ($[Cp^*Rh(bpy)(H_2O)]^{2+}$) being by far the most successful non-enzymatic regeneration catalyst. First reported in 1987 [13,14], it has attracted great interest mainly for its versatility (Scheme 1): (1) in contrast to most chemical reductants known, its catalytically active hydrido form ($[Cp^*Rh(bpy)H]^+$) specifically transfers a hydride ion to the 4-position of $NAD(P)^+$ (due to coordination to the amide-carbonyl-O-atom) thereby exclusively forming the enzymatically active, reduced 1,4-NAD(P)H [15,16]. (2) As a low molecular weight compound, it does not – unlike most enzymatic regeneration systems – distinguish between the phosphorylated and non-phosphorylated nicotinamide cofactor [17,18]. (3) *In situ* regeneration of its catalytically active form is feasible using chemical [18], electrochemical [19], or even photochemical reduction [20–22]. (4) Its catalytic activity is not confined to the regeneration of reduced nicotinamide cofactors. Also *in situ* regeneration of oxidized nicotinamide cofactors [18,23], reductive regeneration of flavins [18,24–27], and heme-groups and analogs [26,28,29] has been reported. (5) Furthermore, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ shows excellent activity and stability over a very broad temperature- and pH range thereby outperforming any enzymatic regeneration system reported [18]. Also, Steckhan and coworkers demonstrated that ligand engineering offers the opportunity of tailoring the catalytic activity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and its derivates [19,30].

Thus, it does not astonish that a broad range of $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed chemoenzymatic and electroenzymatic reductions [23,31–38], oxidations [23] and oxyfunctionalization [24,25,39,40] reactions have been reported.

Compared to enzymatic cofactor regeneration systems such as alcohol dehydrogenases (ADHs), formate- and phosphite dehydrogenase, and NADH oxidases, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ exhibits a somewhat lower catalytic activity in terms of k_{cat} (0.5–10 min^{-1} vs. ca. 100 min^{-1}), which however is compensated by the significantly lower molecular weight resulting in comparable specific activities in terms of U mg^{-1}. Furthermore, its catalytic promiscuity, stability, and robustness to varying reaction conditions is unparalleled by any enzymatic cofactor system known so far.

There is, however, one crucial aspect of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ that severely limits its general applicability for *in situ* regeneration of oxidoreductases, which is the mutually inactivating interaction between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and proteins. While Steckhan and coworkers explicitly ruled out such an interaction at least for the alcohol dehydrogenases from horse liver and *Thermoanaerobium*

* Corresponding author. Tel.: +31 015 2781957; fax: +31 015 2781415.
E-mail address: f.hollmann@tudelft.nl (F. Hollmann).

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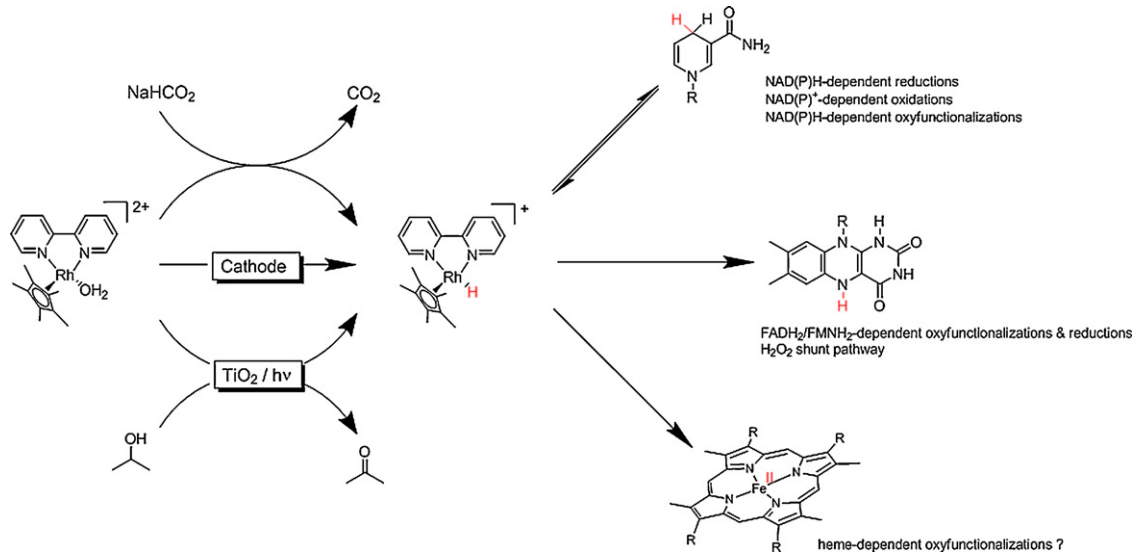


Scheme 1. $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as a versatile catalyst to support chemoenzymatic redox reactions: the catalytically active hydrido species $[Cp^*Rh(bpy)H]^+$ (which can be generated either electrochemically, chemically, or photochemically) can regenerate reduced nicotinamide and flavin cofactors allowing enzymatic redox reactions depending on these cofactors. Also, indications exist that direct regeneration of reduced heme-groups is feasible.

brokii [32], we [41] and others [33,40] have observed this interaction with a range of oxidoreductases. A coordinative interaction between nucleophilic protein residues and the free coordination site at $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (obtained by water displacement) appears to be a plausible mechanism. Tight coordination of protein residues to the 'vacant' Rh-binding site on the one hand would explain the inactivity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as regeneration catalyst under formate-driven conditions. On the other hand, this coordination may be expected to impair enzyme activity if it occurs with catalytically relevant amino acids, blocks the access to the active site, or interferes with the native tertiary and quaternary structure of the enzyme(s).

However, a systematic study investigating this interaction is missing so far. Thus, the controversy about the inactivation has remained rather descriptive and too much relying on assumptions rather than pursuing an in-depth understanding of the underlying inactivation mechanisms.

The aim of the present study therefore was to characterize the inactivation mechanism. As a model enzyme, we chose the recently described alcohol dehydrogenase from *Thermus* sp. ATN1 [42]. Based on these results we propose a range of potential solutions.

Table 1
Residual $[Cp^*Rh(bpy)(H_2O)]^{2+}$-activity (0.02 mM) after 1 h incubation with various enzymes (375 μg ml^{-1}).

Enzyme (source)	Residual activity [%]
None	100
Albumine (bovine serum)	10.8
Acylase I (*Aspergillus mellus*)	17.6
Aldehyde dehydrogenase (*Saccharomyces cerevisiae*)	0
Esterase (*Thermoanaerobium brokii*)	0
Enoate reductase (*Bacillus subtilis*, cell crude extract from *Escherichia coli*)	15.3
Lipase (*Candida rugosa*, Type VII)	13.1
Phytase (*Aspergillus ficuum*)	19.4
Protease (*Aspergillus oryzae*, Type XXIII)	15.3

2. Materials and methods

Unless indicated otherwise, all chemicals were purchased in analytical grade from Sigma–Aldrich and used without further purification. Amino acids were purchased as pure compounds (inner salts).

$[Cp^*Rh(bpy)(H_2O)]^{2+}$ and TADH were synthesized according to published procedures [18,42]. A detailed description can be taken from the supporting information.

Activity assays for both $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and TADH were performed spectrophotometrically based on the formation or depletion of the reduced nicotinamide cofactor (NADH, λ_{max} = 340 nm, ε = 6.22 mM^{-1} cm^{-1}). A detailed description of the experimental details (pH, *T*, etc.) can be taken from the supporting information.

For cyclic voltammetry, the electrochemical cell and conditions reported by Hagen was used [43].

3. Results

3.1. Interaction of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with different enzymes

From previous investigations, a rather diverse picture about the interaction of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with proteins evolves. To clarify whether this interaction is more likely the rule or rather the exception, we incubated $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with different enzymes from various enzyme classes and organisms and determined the residual $[Cp^*Rh(bpy)(H_2O)]^{2+}$ activity in the formate-driven reduction of NAD^+ (Table 1). This reaction represents a simple and sensitive probe for coordination strength of ligands to the Rh-central atom as strongly coordinating ligands impede the coordinative interaction of formate and the Rh-central atom [15,16].

With all enzyme preparations tested, a significant reduction of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ activity was observed, which we attribute to the coordinative interaction between the enzymes and $[Cp^*Rh(bpy)(H_2O)]^{2+}$. It should, however, be mentioned here that not in every case, detailed information about the enzyme formulation was available. Thus, in some cases decreased $[Cp^*Rh(bpy)(H_2O)]^{2+}$-activity may also have been caused by inactivating buffer components (vide infra).

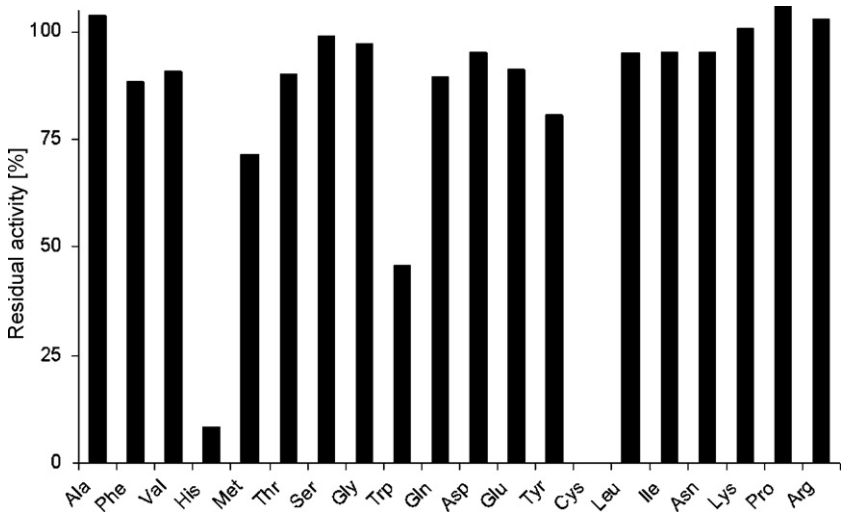


Fig. 1. Residual catalytic activity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ after pre-incubation with amino acids (1 equiv.).

Given the heterogeneous nature of enzyme classes and protein folds tested, we suspect that the interaction of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with proteins is rather a general phenomenon than an exception.

3.2. Interaction of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with amino acids

Next, we investigated the influence of various natural amino acids on the efficiency of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ for the formate-driven reduction of NAD^+. As shown in Fig. 1, a significant reduction of activity (residual activity in parentheses) was observed only in the presence of histidine (8.2%), methionine (71.2%), tryptophane (45.9%), and cysteine (0%). Thus, we conclude that under physiological pH values the protolysis grade of α-amino functionalities (pK_a = 9.2–10.6) and of potentially coordinating OH-groups (serine and threonine) as well as amines (lysine, pK_a = 10.5) and amides/guanidines (asparagine, glutamine, arginine, pK_a ca. 12.5) is not high enough to quantitatively coordinate to the Rh-central atom. Also steric effects and/or differences in nucleophilicity may play a role. The strong inactivating effect of histidine becomes clear considering the relative high ratio (ca. 90%) of non-protonated imidazolium over protonated at pH 7 (pK_a = 6). The somewhat lower effect of tryptophane might have been due to the lower basicity on the one hand and greater steric constraints on the other hand. Interestingly, cysteine which should be near-completely protonated at pH 7 showed the most significant interaction with $[Cp^*Rh(bpy)(H_2O)]^{2+}$, probably due to the high thiophilicity of the soft heavy metal.

The kinetics of this interaction was fast as exemplified with cysteine. $[Cp^*Rh(bpy)(H_2O)]^{2+}$ lost approx. 80% of its initial NAD^+-reduction activity after only 3 min and full inhibition was observed within less than 1 h after addition of cysteine (supporting information). These kinetics were corroborated by the spectral changes, which $[Cp^*Rh(bpy)(H_2O)]^{2+}$ underwent when incubated with cysteine (supporting information). Characteristic absorption changes at 270 and 340 nm were observed and correlated well with the time-course of decreasing catalytic activity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (supporting information). Similar rapid kinetics was also observed for histidine and tryptophane (data not shown).

Interestingly, if the contact time between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and cysteine or histidine was very short (i.e. when addition of these amino acids to the assay mixture occurred immediately before the addition of formate) a significant reduction of the apparent Michaelis–Menten constant (K_M) for formate was observed (Fig. 2). In the absence of either amino acid K_M (formate) was 93 mM whereas this value dropped to 5 mM and 37 mM in the presence of 1 equiv. of cysteine and histidine, respectively. In both cases the maximum activity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (v_{max}, under the given conditions 35 h^{-1}) did not change. The axis interceptions for the single Lineweaver–Burk plots were practically identical suggesting a 'competitive activation' of $[Cp^*Rh(bpy)(H_2O)]^{2+}$. The Michaelis–Menten behavior in the presence of non-coordinating amino acids such as alanine was super imposable to the curve without any additional ligands (data not shown).

The stoichiometry of the interaction between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and the inhibiting amino acids was examined as well (Fig. 3). Thus, for cysteine, a clear 1:1 ratio was observed while for histidine and tryptophane full inhibition was achieved at 1:1.5 and 1:>2, respectively, reflecting the relative degree of $[Cp^*Rh(bpy)(H_2O)]^{2+}$-inhibition shown in Fig. 1. We attribute the apparent deviation from equimolar stoichiometry in case of histidine and tryptophane to lower coordination strength of these ligands rather than to deviations from the assumed 1:1 stoichiometry. Further studies however will be necessary to substantiate this assumption.

3.3. Interaction of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with TADH

The interaction of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with isolated amino acids is only a very restricted model for the mutual inactivation

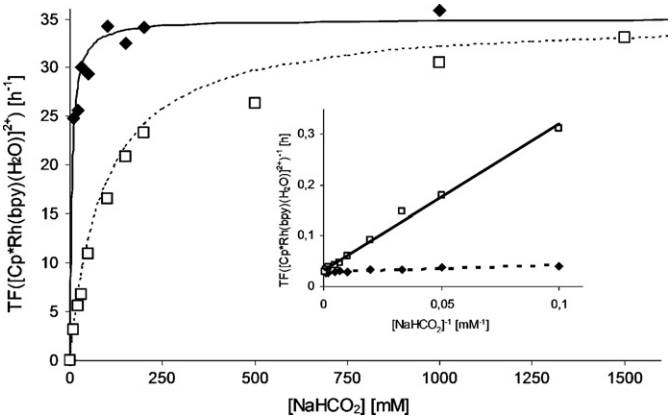


Fig. 2. Michaelis–Menten behavior of formate in the formate-driven reduction of NAD^+ in the presence (♦) and absence (□) of cysteine. Inset: Lineweaver–Burk plot.

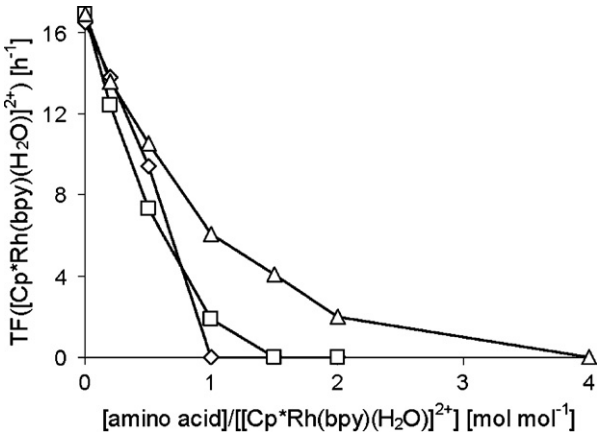


Fig. 3. Stoichiometry of the cysteine- (◊), histidine- (□), and tryptophane (△)-related inhibition of $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

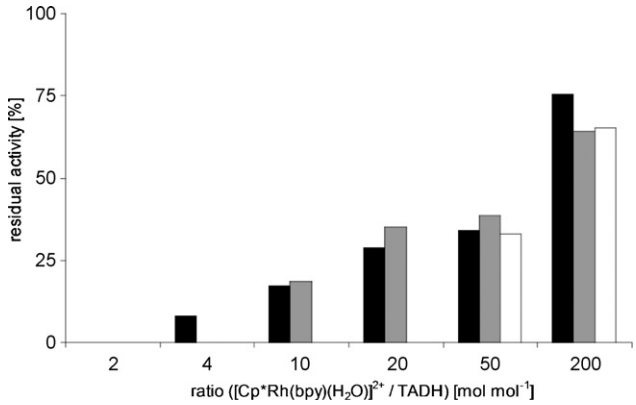


Fig. 4. Residual $[Cp^*Rh(bpy)(H_2O)]^{2+}$-activity after incubation with TADH at different molar ratios and incubation times: 1 h (black), 4 h (grey), 24 h (white).

observed between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and proteins. For example, pK_a values can be significantly modulated by the local electrostatic potential within an enzyme. We therefore further investigated the interaction between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and the recently described alcohol dehydrogenase from *Thermus* sp. ATN1 (TADH) [42]. This enzyme appeared a suitable model system for various reasons. On the one hand fermentative production at scale is straightforward in *Escherichia coli* as host system expressing TADH encoded on a pET vector [42] and using the autoinduction system reported by Studier [44]. Furthermore, one-step purification via heat-treatment of harvested *E. coli* yielded sufficiently pure protein (>95% as judged by SDS gel analysis) to exclude perturbing interactions with host proteins and other components such as protein stabilizers.

In a first set of experiments we determined the residual activity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ in the presence of different molar ratios with TADH and different incubation times (Fig. 4).

The degree of $[Cp^*Rh(bpy)(H_2O)]^{2+}$-inhibition correlated both with the molar ratio to TADH, and incubation time. Up to 4 equiv. of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ were quickly (within minutes) and quantitatively inactivated suggesting the presence of four easily accessible '$[Cp^*Rh(bpy)(H_2O)]^{2+}$-binding sites' per subunit interacting with the metal center. Upon prolonged incubation at least 20 equiv. of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ were inactivated by TADH. The incubation temperature slightly influenced the degree of inhibition. For example performing the incubation experiments at 40 °C instead of ambient temperature resulted in an increased inactivation by approximately 10% (data not shown).

The effect of varying $[Cp^*Rh(bpy)(H_2O)]^{2+}$ concentrations on the activity of the biocatalyst is shown in Fig. 5.

To our surprise, no decrease in TADH activity was observed until a $[Cp^*Rh(bpy)(H_2O)]^{2+}$ to TADH ratio of up to 4. Furthermore, the thermal stability of TADH (in terms of half life time at 60 °C) was not impaired by the presence of 4 equiv. of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (data not shown). Thus, we assume that these four, probably surface-exposed, metal binding sites are not crucial for TADH activity or -stability. Increasing the relative amount of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ to TADH resulted in decreased residual enzyme activity. Interestingly, the temperature-dependency of the inactivation was not uniform. Raising the incubation temperature from 25 °C to 40 °C did not significantly influence the degree of TADH inactivation. At 60 °C, however, TADH inactivation after 1 h was significantly higher, at least for $[Cp^*Rh(bpy)(H_2O)]^{2+}$ to TADH ratios higher than 10 (Fig. 5). This inactivation is accompanied by significant changes in the circular dichroism spectrum of TADH at 270 and 300 nm indicating loss of secondary structural elements (supporting information). It should be mentioned here that the experiments shown in Fig. 5 were performed at pH 6 representing the optimal pH range for TADH-catalyzed reduction reactions. Therefore, direct compari-

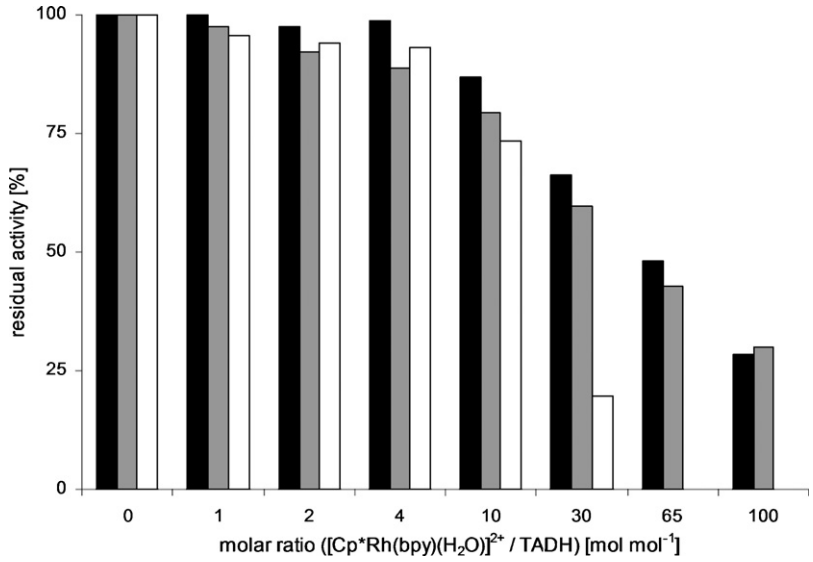


Fig. 5. Residual TADH activity after 1 h incubation with $[Cp^*Rh(bpy)(H_2O)]^{2+}$ at 25 °C (black), 40 °C (grey), 60 °C (white).

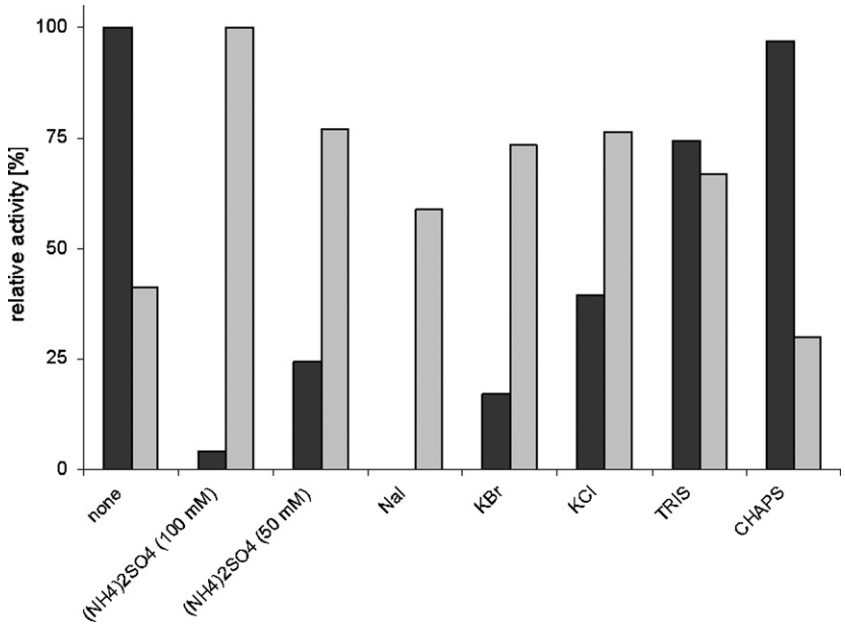


Fig. 6. Effect of various coordinating components on the residual $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (black) and TADH activity (grey).

son with the previous experiments (Fig. 4) may not be possible due to differences in proteolysis degree of crucial amino acids involved. However, we observed a significant stabilization of TADH towards $[Cp^*Rh(bpy)(H_2O)]^{2+}$ when performing the incubation experiments at pH 8. For example, TADH retained more than 80% of its initial activity at pH 8 while at pH 6 only 25% were recovered (in both cases 1 h incubation at ambient temperature, 100-fold molar excess of $[Cp^*Rh(bpy)(H_2O)]^{2+}$). We hypothesize that the deprotonated hydroxyl complex ($[Cp^*Rh(bpy)(OH)]^+$, $pK_a = 8.5$) containing the poor leaving group OH^- interacts to a much lesser extent with nucleophilic protein residues than the aquo complex ($[Cp^*Rh(bpy)(H_2O)]^{2+}$). This led us to further investigate the influence of some potentially strongly coordinating additives on the stability of TADH in the presence of $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

3.4. On the stability of TADH in the presence of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and various coordinating buffers

It is well known that various N- or S-containing compounds can strongly coordinate to Rh. We hypothesized that such coordinative saturation of the Rh-central atom might stabilize TADH (and other proteins in general) towards $[Cp^*Rh(bpy)(H_2O)]^{2+}$. Therefore, we screened a range of putative good coordinating compounds such as nitrogen-containing buffers and halogenides (supporting information) on their effect on TADH-stability in the presence of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (Fig. 6). A clear correlation between their inhibiting effect on formate-driven, $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed NAD^+ reduction and residual TADH activity was observed.

To probe the reversibility/irreversibility of $[Cp^*Rh(bpy)(H_2O)]^{2+}$-related inhibition of TADH we performed a series of competition experiments. Thus, TADH-samples treated with $[Cp^*Rh(bpy)(H_2O)]^{2+}$ were supplemented with 100 mM $(NH_4)_2SO_4$ after 2 and 24 h, respectively. After 2 h, almost full initial TADH activity could be restored upon $(NH_4)_2SO_4$ addition. If however, this treatment was initiated after 24 h TADH activity could not be restored anymore. Furthermore, after 24 h incubation in the presence of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ a yellow, protein-containing precipitate was observed. Obviously, at relatively short incubation times TADH is reversibly inhibited while after prolonged incubation times it is irreversibly inactivated.

3.5. Cyclic voltammetry

On the one hand strongly coordinating buffers stabilize TADH towards $[Cp^*Rh(bpy)L]^{n+}$ (with L being the ligand) but, on the other hand inactivate the metal complex towards formate-driven regeneration of NADH. However, the catalytic hydrido species ($[Cp^*Rh(bpy)H]^+$) can also be formed electrochemically. According to Steckhan et al. [19] this should also be possible in the presence of strongly coordinating buffers (supporting information). Therefore, we investigated the electrocatalytic activity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ equilibrated in $(NH_4)_2SO_4$-containing buffer (Fig. 7).

The increase of cathodic peak current in the presence of NAD^+ (catalytic effect) indicates that indeed the electrochemical formation of $[Cp^*Rh(bpy)H]^+$ and its reaction with NAD^+ is not inhibited by the presence of NH_3. Similarly, catalytic effects were observed also in the presence of Cl^- and TRIS (supporting information).

Overall, we conclude, that strongly coordinating components can prevent $[Cp^*Rh(bpy)(H_2O)]^{2+}$-related enzyme inactivation while preserving its activity for electrochemical NADH regeneration.

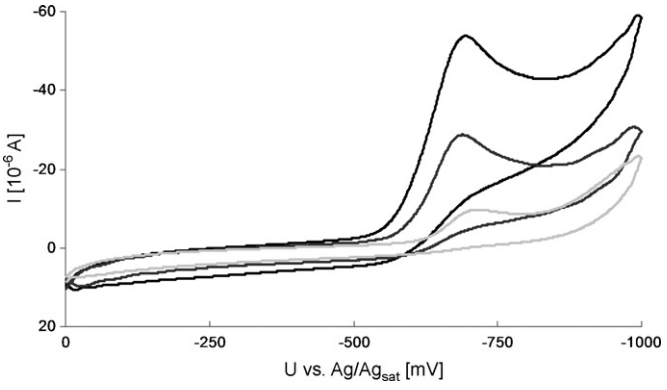
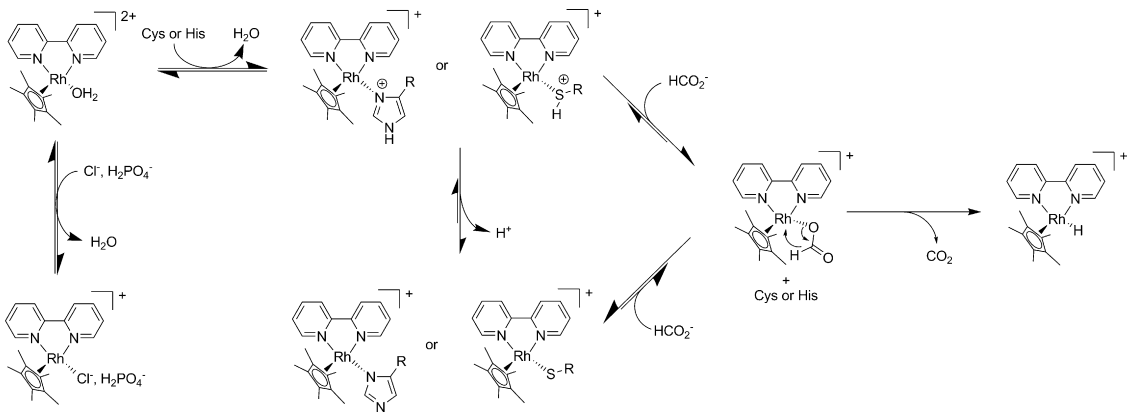


Fig. 7. Cyclic voltammogram of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (overnight equilibrated in 100 mM $(NH_4)_2SO_4$), without NAD^+ (light grey), in the presence of 1 equiv. NAD^+ (dark grey), and in the presence of 2 equiv. NAD^+ (black).



Scheme 2. Proposed coordination/deprotonation mechanism explaining the activating effect of cysteine and histidine at short incubation times and the inhibiting effect at prolonged incubation times.

4. Discussion

$[Cp^*Rh(bpy)(H_2O)]^{2+}$ is a useful catalyst for the *in situ* regeneration of various oxidoreductase cofactors and prosthetic groups (Scheme 1) which is well-documented by its various applications. Less well-documented however is that it has sometimes a detrimental effect on enzyme stability let alone the underlying inactivation mechanism. Aim of this study was to shed some light on this inactivation effect.

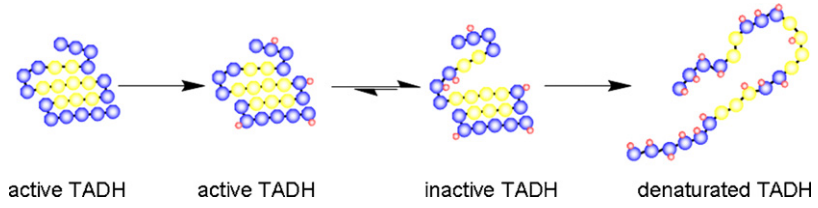
4.1. The interaction between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and amino acids

Inspired by the study by Hildebrand and Lütz [33] we validated their observation that among the isolated amino acids primarily cysteine, histidine, and tryptophane (and methionine albeit to a lower extend) exceed an inhibiting effect on $[Cp^*Rh(bpy)(H_2O)]^{2+}$ suggesting these amino acids to be the coordinating residues within a protein (Fig. 1). Especially cysteine and histidine had a very interesting effect on $[Cp^*Rh(bpy)(H_2O)]^{2+}$ activity. After prolonged incubation, these 'ligands' severely impaired the formate-driven reduction of NAD^+ which we attribute to the formation of a tight (thermodynamically and kinetically stable) bond to the Rh-central atom thereby preventing formate coordination. But at very short incubation times, these amino acids exhibited an activating effect in the sense that the affinity of formate to Rh (expressed as K_M value) was drastically increased (Fig. 2). These observations may be rationalized by the hypothetical coordination equilibrium outlined in Scheme 2.

The protonated amino acids represent the primary products in the coordination reaction to the Rh-central atom. After deprotonation, tight Rh–S and Rh–N bonds are formed showing no exchange tendency with, e.g. formate (or protein residues) and thereby yielding catalytically inactive complexes; i.e. formate cannot substitute the tightly binding ligands. If however the time-scale for deprotonation is too short (less than 1–2 min), formate competes with the less strongly binding, protonated amino acids for coordination to the central atom. Compared to the water ligand, these protonated amino acid species are better leaving groups explaining that v_{max} is reached at lower formate concentrations. It should be emphasized here that this activating effect can only be observed at short contact times between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and the amino acid(s). At prolonged incubation times deprotonation of His and Cys occurs resulting in thermodynamically and kinetically stable adducts with which substitution of the ligand by formate or protein residues does not occur. In other words, the K_M value for other ligands has increased dramatically.

4.2. The interaction between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and TADH

The results presented here reveal that a simple stoichiometry for the interaction between TADH and $[Cp^*Rh(bpy)(H_2O)]^{2+}$ cannot be made. At least 20 $[Cp^*Rh(bpy)(H_2O)]^{2+}$-binding events were observed in TADH/$[Cp^*Rh(bpy)(H_2O)]^{2+}$ titration experiments (Fig. 4) but more may be assumed. TADH contains in total 9 histidines and 5 cysteines (supporting information), which do not explain the observed 'stoichiometry' of at least 20. Quantitative analysis is complicated by the only partial inhibition by amino acids such as methionine, tryptophane (Fig. 1). Therefore, we made a rough estimation taking into account all amino acids present in TADH. The contribution of each amino acid to $[Cp^*Rh(bpy)(H_2O)]^{2+}$-inhibition was weighted by its influence on $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as isolated amino acid (Fig. 1). Thus, an overall numerical stoichiometry of 1:24.2 (TADH/$[Cp^*Rh(bpy)(H_2O)]^{2+}$) was estimated which is in good accordance with the results obtained in Figs. 4 and 5. Of course this suggests an interaction of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with every (or most) amino acid residue(s) within TADH, which can only occur if the protein is fully unfolded. Two to four of these binding sites seem to be surface-exposed as judged by the fast inhibition of up to 4 equiv. of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ within 1–2 h. Interestingly, these interactions did not impair TADH activity. Unfortunately, at present no crystal structure or homology model of TADH is available to further rationalize this stoichiometry. Further $[Cp^*Rh(bpy)(H_2O)]^{2+}$-binding occurred at a lower rate and showed



Scheme 3. Proposed mechanism for the $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed inactivation of TADH. Blue: hydrophilic amino acid residues, yellow: hydrophobic amino acid residues, red: $[Cp^*Rh(bpy)(H_2O)]^{2+}$. (For interpretation of the references to colour in this scheme, the reader is referred to the web version of the article.)

a detrimental effect on TADH activity. It is interesting to note that raising the incubation temperature from ambient temperature to 40 °C had only a minor effect on TADH inhibition. If however, the incubation temperature was raised to 60 °C, TADH inhibition was much more pronounced (Fig. 5). 60 °C is the optimal growth temperature of *Thermus* sp. ATN1 [42], the original host organism. Thus, it may be expected that TADH exhibits high conformational flexibility at this temperature to function optimally in its native environment while it is more rigid at lower temperatures. Therefore we hypothesize a '$[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed' unfolding of TADH (Scheme 3).

This mechanism is based on the generally accepted mechanism for thermal inactivation of proteins comprising: (1) the catalytically active enzyme is in a reversible equilibrium with an inactive, partially unfolded structure. Thus, hydrophobic amino acids, normally located in the protein core are temporarily surface-exposed. (2) These hydrophobic residues can agglomerate to reduce their thermodynamically unfavorable solvent exposure yielding the irreversibly denaturated enzyme [45]. According to this mechanism, we propose that the binding of two to four $[Cp^*Rh(bpy)(H_2O)]^{2+}$ influenced neither the catalytic activity of TADH nor the equilibrium between active and partially unfolded protein (neither TADH's catalytic activity nor its thermal stability was impaired in the presence of up to 4 equiv. of $[Cp^*Rh(bpy)(H_2O)]^{2+}$). However, further binding of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ to the partially unfolded enzyme stabilized this form, possibly by blocking essential inter-protein interaction and/or by electrostatic repulsion. Thus, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ binding shifts the equilibrium between active and partially unfolded protein and thereby accelerates TADH denaturation. This assumption is further supported by the time-dependent switch from reversible inhibition to irreversible inactivation of TADH ($(NH_4)_2SO_4$ treatment could restore TADH activity after 2 h but not after 24 h).

This mechanism would also explain the stabilization observed for 2-hydroxy biphenyl 3-monooxygenase (HbpA) immobilized on Eupergit [45]. Here, multiple-point attachment of the enzyme to the carrier might have stabilized the catalytically active tertiary and quaternary structure of the enzyme against $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

4.3. Potential remedies

Based on the observations reported in this contribution we discuss a range of potential solutions to the mutual inactivation of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with enzymes. One obvious solution was reported by Lütz and coworker [33]. Physical separation of the enzyme(s) and $[Cp^*Rh(bpy)(H_2O)]^{2+}$ was shown to greatly stabilize both. However, diffusion limitation severely limited the catalytic performance of the single components in terms of turnover frequency and total turnover number of the catalysts.

Enzyme engineering to generate inert variants does not seem viable considering the multiplicity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ binding sites within TADH. Exchanging all of these residues for sure is tedious and most probably results in catalytically inactive/impaired TADH variants. Furthermore, this would represent just a case-specific solution lacking general applicability.

Therefore, we propose to further explore strongly coordinating ligands to protect the enzyme(s) from $[Cp^*Rh(bpy)(H_2O)]^{2+}$-related inactivation. For example ammonium containing buffers proved to be very efficient to stabilize TADH (Fig. 6) while electrocatalytic NAD(P)H regeneration activity of $[Cp^*Rh(bpy)(NH_3)]^{2+}$ (at present we can only speculate on the exact nature of the ammonium adduct, possibly NH_3 is deprotonated in the Rh-ligand sphere yielding $[Cp^*Rh(bpy)(NH_2)]^{+}$) was not impaired (Fig. 7). The contradictory indications from the literature reporting stabilizing effects [31,41] and no effect [33] necessitate further in-depth studies to clarify the possibility of a stabilizing effect. However, the results reported here might eventually lead to robust and efficient electroenzymatic reduction reactions.

Further studies will be necessary to establish a general mechanism explaining the role of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ in enzyme inactivation. However, the data presented here give a strong indication that its major role lies in the stabilization of unfolded, catalytically inactive states. If so, stabilization of these structural elements, e.g. via multiple-point crosslinking and/or immobilization would represent another simple solution to the inactivation problem. Then, coordinative saturation of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ would not be necessary to stabilize the enzyme (as it would be geometrically stabilized) and *en masse* inactivation of the metal complex would not occur as the majority of binding amino acids would remain inaccessible in the protein core. Again, the contradictory results reported [33,41] on this require further investigation to clarify this issue.

5. Conclusions

In the present contribution we have, for the first time, performed an in-depth analysis of the mutually inactivating interaction between $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and enzymes. Based on the stoichiometric and kinetic analysis, we hypothesize an inactivation mechanism and propose several remedies.

These studies will put the basis for various future developments using $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.01.006.

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