Photo-Driven Hydrogen Evolution by an Artificial Hydrogenase Utilizing the Biotin-Streptavidin Technology

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Photocatalytic hydrogen evolution by an artificial hydrogenase based on the biotin-streptavidin technology is reported. A biotinylated cobalt pentapyridyl-based hydrogen evolution catalyst (HEC) was incorporated into different mutants of streptavidin. Catalysis with [Ru(bpy)3]Cl2 as a photosensitizer (PS) and ascorbate as sacrificial electron donor (SED) at different pH values highlighted the impact of close lying amino acids that may act as a proton relay under the reaction conditions (Asp, Arg, Lys). In the presence of a close-lying lysine residue, both, the rates were improved, and the reaction was initiated much faster. The X-ray crystal structure of the artificial hydrogenase reveals a distance of 8.8 Å between the closest lying Co-moieties. We thus suggest that the hydrogen evolution mechanism proceeds via a single Co centre. Our findings highlight that streptavidin is a versatile host protein for the assembly of artificial hydrogenases and their activity can be fine-tuned via mutagenesis.

Keywords: artificial hydrogenase • hydrogen evolution • photosensitizer • biotin-streptavidin

Introduction

Splitting water into O2 and H2 using sunlight as energy source offers an attractive means to address the World’s growing energy needs.[1–4] Clean energies including solar, tidal, wind- or hydroelectric-power plants are widely used, but generate electricity which is challenging to store. Storing the energy within chemical bonds and producing an energy carrier that can be liquefied or transported as a gas is desirable. There are multiple ways of producing hydrogen on an industrial scale, yet most of them rely on carbon sources, precious metal catalysts and are not energy efficient.[5–7] Hydrogenases are natural enzymes found in a variety of bacterial and archaeal organisms, where they generate reducing equivalents in the form of H2 or oxidize H2 in reactions that require a reductant.[8–11] Such enzymes are used in reactions including: olefin metathesis, Suzuki-coupling, C–H activation or transfer hydrogenation etc.[30,34–37]

The biotin-streptavidin technology has found widespread use for the assembly of artificial metalloenzymes (ArMs). [27–33] Streptavidin (Sav) is a versatile host protein for the assembly of homogeneous Co-based hydrogen-evolution catalysts, we selected this base technology have been developed to catalyze a wide variety of water compatible reactions including: olefin metathesis, Suzuki-coupling, C–H activation or transfer hydrogenation etc.[10,14–37]

Scheme 1. Synthetic pathway of the hydrogen evolution catalyst [CoBr(appy)-OH]Br and its biotinylated counterpart [CoBr(appy)-biot]Br (biotin depicted in blue, see the supporting information for details).

Herein we report on our efforts to engineer an artificial hydrogenase based on the biotin-streptavidin technology. In view of the versatility of homogeneous Co-based hydrogen-evolution catalysts, we selected this base metal for the development of ArMs using [Ru(bpy)3]Cl2 as photosensitizer and ascorbate as reducing agent.
Results and Discussion

Synthesis and binding studies
The pentapyridyl (appy) ligand and the corresponding Co-catalyst [CoBr(appy)-OH]Br were selected for derivatization with a biotin anchor, Scheme 1. The biotinylated hydrogen evolution catalyst (HEC) [CoBr(appy)-Biot]Br was synthesized in 6-steps from commercial compounds, (Scheme 1, Figures S1-S6, see SI for details). The hydroxy-bearing pentapyridine (appy)-OH (1) was reacted with 1-iodo-3-azido-propane (2) to afford the corresponding azido ether (appy)-N3 (3). The azide group was reduced and the resulting amine (appy)-NH2 (4) was coupled to activated biotin-pentafluorophenylester (5) to afford the biotinylated ligand (appy)-Biot (6). Reaction with CoBr2 in MeOH yielded [CoBr(appy)-Biot]Br as a light brown solid which was purified by recrystallization. The affinity of [CoBr(appy)-Biot]Br towards streptavidin was evaluated relying on a displacement titration using 2-(4-hydroxyphenylazo)benzoic acid (HABA).

Photocatalysis and genetic optimization
To compare the activity of the Co-precursor [CoBr(appy)-OH]Br and its biotinylated analogue [CoBr(appy)-Biot]Br, argon-flushed, buffered (pH = 5) aqueous solutions (10 ml total volume) were charged with 5 µM catalyst concentration, 1 M AscOH (sacrificial electron donor), 100 µM [Ru(bpy)3]Cl2 (photosensitizer) and were illuminated with a LED (453 nm and 0.3 µE s-1). Hydrogen evolution was continuously monitored by an automated GC as described elsewhere.

With no Sav present, both [CoBr(appy)-Biot]Br and [CoBr(appy)-OH]Br display similar turnover numbers after 6.5 hours (TON = 1050 and TON = 1120 after 6 hours, black and blue traces for [CoBr(appy)-Biot]Br and [CoBr(appy)-OH]Br respectively, Figure 1a). Addition of Sav WT (10 µM free biotin binding sites, corresponding to 2.5 µM tetrameric Sav) did not impact the activity of [CoBr(appy)-OH]Br, highlighting that there are no detrimental, non-specific interactions between the cofactor and Sav (grey trace, Figure 1a). In contrast, the biotinylated HEC, bound to SavWT, displayed a marked decrease in activity (TON = 820 after 6 hours) highlighting the importance of second coordination sphere interactions on hydrogenase activity (green trace, Figure 1a).

The leveling of the activity after 3-4 hours is owed to the accumulation of dehydroascorbic acid (DHA), the oxidized form of ascorbic acid. As shown before, addition of TCEP regenerates ascorbate, and H2 production of [CoBr(appy)-OH]Br was significantly prolonged, in many cases until full conversion of TCEP was achieved. Additionally, the Sav bound catalyst should be diffusionally- and sterically hindered as compared to [CoBr(appy)-OH]Br, thus slowing the rate of electron transfer from photogenerated, reduced photosensitizer. This, in turn, results in a higher sensitivity of the Sav system for back electron transfer to DHA, due to a higher steady state concentration of reduced
photosensitizer. Thus, we set out to explore the impact of the second coordination sphere of Sav. For this purpose, a focused library of Sav single point mutants was screened in combination with [CoBr(appy)-Biot]Br. Building on previous studies, two close lying positions were selected: S112 and K121. Substitution of the serine residue S112 by either an Asp (Sav S112D, TON = 1070 after 6 hours, light green trace, Figure 1b) or a Arg (Sav S112R, TON = 970 after 6 hours, turquoise trace, Figure 1b) lead to an increase in TON compared to Sav WT (green trace, figure 1b). We speculate that this might be due to the presence of close lying amino acids capable of acting as a proton relay, potentially facilitating outer-sphere protonation of the Co-H species, as suggested in related studies.[43–48] Thus an increase in the rate of H₂ formation at the ArM, and a concomitant removal of reducing equivalents from the system, would be expected. That way, back electron transfer to DHA would be prevented to a certain level, in line with the observed increase in both, rate and TON. This was confirmed by the improved activity of the S112K mutant (Ser → Lys) for which the highest activity was observed (TON = 13 nmol H₂/s (black squares, Figure 1c) and the incorporated cofactor [CoBr(appy)-Biot]Br has a maximum rate of 9 nmol H₂/s (blue triangles, Figure 1c), the non-biotinylated HEC [CoBr(appy)-OH]Br peaks at 13 nmol H₂/s (black squares, Figure 1c) and the incorporated cofactor [CoBr(appy)-Biot]Br • Sav S112K peaks at 12 nmol H₂/s (red triangles, Figure 1c). The presence of a proton relay in the immediate proximity of the (appy)-moiety affects both the TON and the corresponding rates. The effect of the additional lysine is highlighted by the use of Sav WT (green triangles, Figure 1c) and the worst performing mutant K121W (orange squares, Figure 1c). In fact, since the binding pocket is symmetrical, not only two lysines are present within the binding pocket (S112K and K121) but four (2xS112K and 2xK121, Figure 5). This finding suggests that polar groups surrounding the catalytic centre can serve as proton relays and are indeed essential for its reactivity. When the polar groups are substituted by apolar groups as alanine the reactivity drops significantly.

pH Dependence

Hydrogen evolution at neutral pH is desirable, because it would allow the use of fresh or even sea water.169 Since the protein provides a second coordination sphere around the catalytic centre and lysines in the close proximity of the cofactor have a positive effect on catalytic performance, we anticipated a marked pH influence on catalysis. A pH screening of [CoBr(appy)-OH]Br, [CoBr(appy)-Biot]Br and [CoBr(appy)-Biot]Br • Sav S112K was performed (Figures S8-S13 and Tables S1 and S2). At pH 4 and pH 5 in 1 M AscOH buffer, [CoBr(appy)-OH]Br shows higher maximal rates than the biotinylated HEC [CoBr(appy)-Biot]Br either inside or outside the Sav S112K (Figure 2). Increasing the pH above 5.0, leads to an inversion in trend: at pH 5.7 (using 1 M acetate buffer with 0.1 M AscOH) the hydrogen production rates follow the following order: [CoBr(appy)-Biot]Br • Sav S112K > [CoBr(appy)-OH]Br (Figure 2).

Incorporation of the biotinylated cofactor [CoBr(appy)-Biot]Br within Sav S112K affects both the reaction rate as well as the time required to reach the maximum rate (Figure 3). At pH 7.5, [CoBr(appy)-Biot]Br • Sav S112K reaches after 500 minutes its maximum rate, whereas for [CoBr(appy)-OH]Br, 1100 minutes are required. Monitoring both the rate and TON at pH = 7.0 reveals that [CoBr(appy)-Biot]Br • Sav S112K outperforms both [CoBr(appy)-OH]Br and [CoBr(appy)-Biot]Br, Figure 4. The most striking difference, apart from the increase in rate, is the shorter time required until the maximum rate is reached. In line with the arguments from the previous section, protic residues on Sav could increase the rate of hydrogen formation. Thus, a lower steady state concentration of reduced HEC is expected, lowering the probability for deleterious back electron transfer, and increasing the hydrogen formation rate from the beginning.
**Figure 3.** Summary of times required to reach the maximum rate of hydrogen production as a function of pH. [CoBr(appy)-Biot]Br • Sav S112K (red triangles), [CoBr(appy)-OH]Br (black triangles) and [CoBr(appy)-Biot]Br (blue triangles).

To rule out the possibility of spurious Co(II) species acting as catalyst, control experiments were conducted whereby some of the catalyst’s components were omitted (Figure S14). Compared to [CoBr(appy)-Biot]Br • Sav S112K, under otherwise identical reaction conditions, Ru(bpy) 32+ alone, Sav S112K, CoBr2 or CoBr 2 • Sav S112K yielded only traces of hydrogen. These observations suggest that the pentapyridine-coordinated Co(II) is indeed the active species generating H2 from the Co–H moiety.

**Figure 4.** Comparison of the hydrogen production rate (left scale) and TON (right scale) for [CoBr(appy)-OH]Br (black trace), [CoBr(appy)-Biot]Br (blue trace) and [CoBr(appy)-OH]Br • Sav S112K (red trace) upon irradiation at 453 nm with 100 µM [Ru(bpy) 3]Cl2 in 1 M NaHPO4 buffer (pH 7) and 0.1 M AscOH present (See SI for details).

**Structural Insight**

The localization of CoBr(appy)-Biot]Br within streptavidin was scrutinized by X-ray crystallography. A crystal of complex [CoBr(appy)-Biot]Br • Sav-K121A was obtained upon soaking the apoprotein crystals with an excess of cofactor overnight. The X-ray structure of [CoBr(appy)-Biot]Br • Sav S112A was solved to 1.7 Å resolution (Figures 5 , S15 and Table S3). Upon solving the protein structure by molecular replacement, residual electron density was visible in the biotin binding pocket. Complex [CoH2O(appy)-Biot]2+ was modelled in each of the four Sav monomers with full occupancy and in a single conformation. The first coordination sphere around cobalt is virtually identical to that found in the corresponding small molecule crystal structure (CCDC identifier: LILYAI).

**Figure 5.** Close-up view of the active site in the crystal structure of complex [CoH2O(appy)-Biot] • Sav-K121A (PDB code 6FRY). The protein is displayed as surface and cartoon model and the cofactor as stick model. The cofactor is contoured by a 2Fo-Fc electron density map (1.0 σ, blue colour) and an anomalous dispersion density map (3.0 σ, green colour). The orange sphere indicates a cobalt and the magenta stick a water molecule. For clarity only one cofactor molecule is displayed per Sav tetramer.

The central metal is coordinated by the heteroaromatic groups in a distorted octahedral fashion. The axial bromide was replaced by water in the metalloenzyme. The bulky cobalt complex is accommodated within the biotin vestibule that is flanked from three side by loops 3,4; 5,6 and 7,8, the fourth site occupying a symmetry-related cofactor molecule. The axial water ligand is exposed to the solvent and is presumed to be the substrate’s binding site. The distance between two neighbouring Co-atoms is 8.8 Å. Considering the large distance between two catalytically competent metals, we tentatively exclude a catalytic mechanism that proceeds via a dinuclear mechanism. This hypothesis is further supported by the fact that, under typical reaction conditions, only half of the biotin binding sites are occupied with [CoBr(appy)-Biot]Br, albeit with a statistical distribution. Of the two postulated mechanisms put forward for Co-based hydrogenase mimics,[43,51,52] we suggest that the present system operates using a single Co-site.

**Conclusions**

In conclusion, we have demonstrated that incorporation of a biotinylated, molecular hydrogen evolution catalyst into streptavidin mutant S112K as a protein scaffold effects its activity in a positive way. The catalyst embedded within the protein not only showed higher turnover numbers at specific pH values but also higher maximum rates and a shorter time to reach them in most cases. The genetically-engineered additional basic amino acid S112K close to the catalyst is beneficial for activity. We suggest that this close lying pendant base is protonated under the reaction conditions and can thus
facilitate protonation of the Co–H species. This hypothesis is further supported by deletion of all close-lying lysine residues. Accordingly, K121A and K121W derived hydrogenases display significantly reduced activities. Furthermore, the incorporation of the catalyst into a protein cavity prevented two Co-centres to interact with another. Since the protein embedded catalyst displayed similar or, in some cases, better activity than the free catalyst, we suggest that the reaction proceeds via a single Co centre. These findings suggest that such biohybrid catalysts may readily be optimized by genetic means. Future studies will focus on applying directed evolution schemes to improve the hydrogenase activity. Finally, we hypothesize that the host protein may be immobilized on an electrode without perturbing the immediate environment around the metal cofactor.

Experimental Section

Experimental details, synthesis procedures, spectra, tables and additional graphs can be found in the supporting information.

Supplementary Material

Supporting information for this article is available on the WWW under Supplementary Material.

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Author Contribution Statement

S. G. K. performed chemistry and catalysis experiments, analyzed the data and wrote the paper. B. P. helped with the catalytic experiments, discussion of the results, and reviewed the manuscript. T. H. solved the X-Ray data and provided the related figures. R. A. and T. R. W. designed the study, supervised the work, discussed the results and corrected the manuscript. All authors have given approval to the final version of the manuscript.

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