Metalloenzymes

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Highly Efficient and Site-Selective Phosphane Modification of Proteins through Hydrazone Linkage: Development of Artificial Metalloenzymes**

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Transition metal/phosphane complexes are well known to be one of the most successful classes of homogeneous catalysts for several industrial reactions such as hydroformylation and hydrogenation.[1] However, for a large number of transformations existing phosphane-based catalysts lack the desired selectivity, which is a driving force for the continuous development of novel phosphane ligand systems.[2] One bioinspired approach is the creation of hybrid catalysts by the introduction of synthetic catalysts into biopolymers. Covalent or noncovalent merging of transition-metal catalysts with proteins generates the opportunity of combining chemical and genetic methods for performance optimization.[3] Protein-based artificial metalloenzymes employing phosphane ligands have mainly been developed using noncovalent anchoring approaches, using either antibodies raised against a diphosphane/rhodium complex[4] or the very successful biotin-avidin system,[5] mainly developed in the group of Ward[6] and later subjected to directed evolution by the group of Reetz.[7] Reetz et al. also covalently introduced a diphosphane ligand in lipases through a phosphonate linkage, but hydrolytic lability of the linker hampered application of these elegant systems.[8] The drawback of the approaches outlined above is that the protein structure space that can be combined with phosphane ligands is very limited. Herein we report the development of a site-specific covalent anchoring method which will allow the introduction of phosphanes in a wide variety of protein structures, demonstrated by modifying three structurally different proteins with a small library of phosphane ligands.

In seeking to exploit proteins to induce shape selectivity in catalytic reactions, we selected several proteins having different cavity architectures: 1) sterol carrier protein-2-like (SCP-2L) domain of multifunctional enzyme type 2 containing an 18 Å long and 9 Å wide hydrophobic tunnel,[9] 2) photoactive yellow protein (PYP),[10] which exhibits a small hydrophobic pocket,[11] and 3) the sensing blue-light using FAD (BLUF) domain of activator of photopigment and puc expression A (AppA)[12] containing a cleft that binds flavins.[13] Several single-cysteine variants for covalent modification with phosphanes were created by site-directed mutagenesis. Modification of the nucleophilic thiol of a unique cysteine is a widely employed strategy for site-selective bioconjugation.[14] A cysteine can be introduced at virtually any position within a protein structure by site-directed mutagenesis and then selectively modified, using for example, alkyl halides or maleimides. This approach has already been successfully applied for the development of artificial metalloenzymes bearing ligands having donor atoms other than phosphorus.[15]

We recently reported the synthesis of artificial metalloenzymes by direct modification of the unique cysteine moiety of PYP using phosphanes which contain an 1,1′-carbonyldiimidazole (CDI) activated carboxylic acid.[16] However, for SCP-2L and AppA this method lacked the desired chemoselectivity.[17a] Nonprotected phosphane-containing maleimides cannot be synthesized because of the nucleophilic character of the phosphane, which leads to the formation of phosphonium salts and phosphorus ylides.[18] By using borane- and sulfur-protected maleimido phosphanes, highly selective cysteine modification was easily achieved, however, deprotection proved incompatible with or inefficient for these protein constructs.[17b]

Hydrazone formation between a hydrazide and aldehyde or ketone is a common bioconjugation method that proceeds under mild reaction conditions in water.[19] The reaction has also been reported to be compatible with phosphanes,[20] prompting us to explore the use of this method for the bioconjugation of phosphanes. The commercially available cross-linker **1** was used for the cysteine-selective introduction of a hydrazide, affording the protein–**1** product (Scheme 1a, Figure 1).

Upon mixing of the hydrazide-modified proteins with excess diphenylphosphane-containing benzaldehydes **P1**–**P3** under inert atmosphere and stirring overnight, a quantitative conversion into the corresponding phosphane-modified proteins was observed by mass spectroscopy (ES^+; Scheme 1b, Figure 1, and Table 1 entries 1–12). The excess of insoluble phosphane aldehyde was easily removed by centrifugation and subsequent washing with buffer in a centrifugal concentrator. The procedure causes minimal loss of protein, as determined by Bradford assays, resulting in typical modified-

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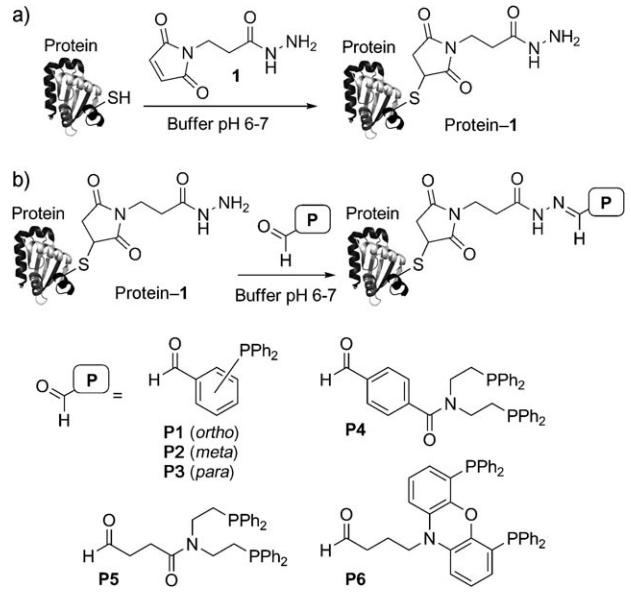

Scheme 1. a) Modification of unique cysteine-containing proteins with hydrazide–maleimide **1**. b) Bioconjugation of hydrazide-modified proteins with phosphane aldehydes **P1**–**P6**.

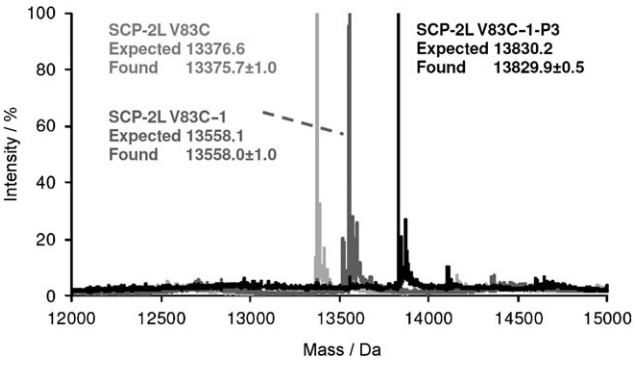

Figure 1. Processed mass spectra (ES^+) of SCP-2L V83C (light gray), SCP-2L V83C–**1** (dark gray), and SCP-2L V83C–**1**–**P3** (black)

protein yields of over 95%. The ^{31}P NMR spectra of PYP–**1** and SCP-2L V83C–**1** modified with **P3** confirmed that the modified proteins contained a free phosphane, showing broad signals at δ values ranging from −2 to −8 ppm (Figure 2 and Figure 3a). In both spectra, the signal appears to consist of at least two overlapping broad peaks. This signal might be the result of the presence of different conformations of the conjugates, caused by either the imine bond, the chiral center formed by the maleimide–sulfide bond, or conformation of the protein. The possibility that the different signals originate from modification of different amino acid side chains of the protein was ruled out by detailed analysis of tryptic digests. Moreover, no protein modification was observed after treatment of unmodified proteins with the phosphane aldehydes. Also by employing Ellman's reagent, the absence of free thiol groups in the conjugates was confirmed upon analysis by mass spectroscopy (ES^+).[21]

The method was extended to diphosphanes **P4–P6**, again affording the desired conjugates with high selectivity,

Table 1: Results of bioconjugation of hydrazide-modified proteins with phosphane aldehydes **P1**–**P6**[a]

Entry	Protein–**1**	P	Calculated mass [Da]	Observed mass [Da][b]
1	SCP-2L V83C–**1**	**P1**	13830.2	13830.4 ± 0.3
2	SCP-2L V83C–**1**	**P2**	13830.2	13830.8 ± 0.6
3	SCP-2L V83C–**1**	**P3**	13830.2	13829.9 ± 0.5
4	PYP–**1**	**P1**	16317.8	16317.0 ± 1.1
5	PYP–**1**	**P2**	16317.8	16318.2 ± 0.8
6	PYP–**1**	**P3**	16317.8	16317.4 ± 0.6
7	AppA Y21C–**1**	**P1**	15850.5	15850.6 ± 0.6
8	AppA Y21C–**1**	**P2**	15850.5	15850.9 ± 0.6
9	AppA Y21C–**1**	**P3**	15850.5	15850.0 ± 0.5
10	AppA Q63C–**1**	**P1**	15885.5	15900.2 ± 0.8[c]
11	AppA Q63C–**1**	**P2**	15885.5	15884.9 ± 0.6
12	AppA Q63C–**1**	**P3**	15885.5	15884.6 ± 0.8
13	SCP-2L V83C–**1**	**P4**	14114.9	14130.1 ± 1.0[c]
14[d]	AppA Y21C–**1**	**P5**	16085.8	16083.4 ± 2.8
15[d]	AppA Q63C–**1**	**P5**	16120.8	16120.2 ± 1.1
16[e]	SCP-2L V83C–**1**	**P5**	14065.3	14065.9 ± 0.7
17[e]	SCP-2LV 83C–**1**	**P6**	14161.8	14163.5 ± 3.0
18[d]	AppA Q63C–**1**	**P6**	16216.9	16216.2 ± 0.9

[a] Reaction conditions: 2–10 equivalents phosphane (**P1**–**P6**) in aqueous buffer pH 6–7; full conversion of protein–**1** was observed unless stated otherwise. [b] Main peak for modified protein. [c] Main peak corresponds to the peak for the protein containing the oxidized phosphane. [d] Some protein–**1** observed. [e] Protein–**1** observed as main peak and only weak signal for phosphane-modified protein–**1** was found.

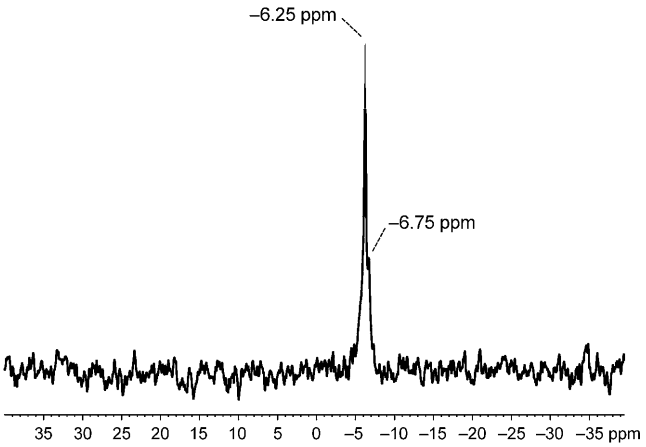

Figure 2. $^{31}P\{^1H\}$ NMR spectrum of PYP–**1** modified with **P3**.

although the signal for the hydrazone-modified proteins was often still be observed (Table 1 entries 13–18). However, conversions were still found to be greater than 90% after correction of the ES^+ data for differences in ionization efficiencies (entries 13–15 and 18). Unfortunately, several of the conjugates containing diphosphanes **P5** and **P6** were prone to protein precipitation, which hampered the determination of the conversion and severely compromised the yield (entries 16 and 17). It appeared that the more reactive phosphane-benzaldehyde-type substrates were more suitable than the aliphatic phosphane aldehydes, as the products were less prone to precipitation. Consequently, typical yields of

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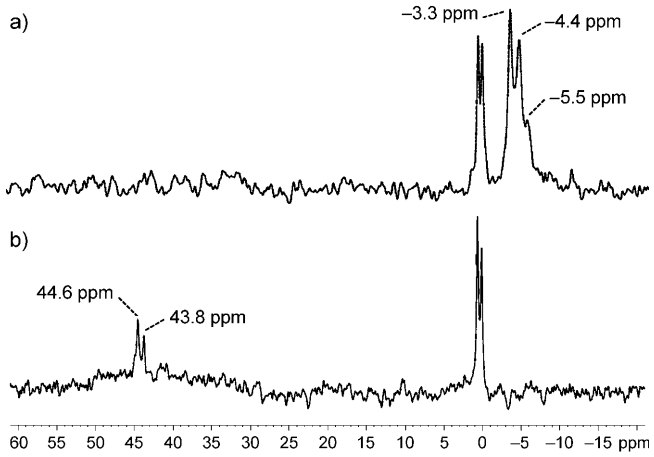

Figure 3. $^{31}P\{^1H\}$ NMR spectra of a) SCP-2L V83C–**1**–**P3**. b) SCP-2L V83C–**1**–**P3** treated with [Rh(acac)$(CO)_2$]. Peaks between $\delta = 2$ and 0 ppm are probably the result of protein phosphorylation as those are also observed in the native protein (see the Supporting Information).

protein–**1**–**P4** (entry 13) were similar to those obtained with **P1**–**P3** (> 95 %).

To demonstrate the formation of protein/phosphane/metal complex, SCP-2L V83C–**1**–**P3** was treated with one equivalent of [Rh(acac)$(CO)_2$] (acac = acetylacetonate). The mass spectrum of the obtained mixture showed a signal corresponding to the SCP-2L V83C–**1**–**P3** modified with a rhodium carbonyl fragment as main peak (Figure 4). The ^{31}P NMR spectrum recorded after the reaction revealed a

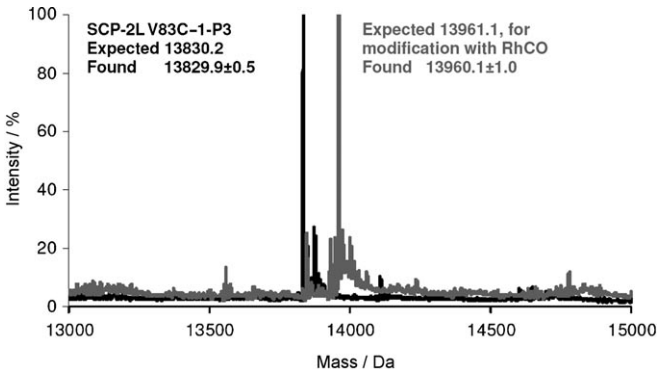

Figure 4. Processed mass spectra (ES^+) of SCP-2L V83C–**1**–**P3** (black) and SCP-2L V83C–**1**–**P3** after treatment with [Rh(acac)$(CO)_2$] (gray).

phosphorus shift of almost 50 ppm to give broad signals between $\delta = 45$ and 43 ppm (Figure 3b). These results indicate successful formation of a rhodium phosphane complex attached to the protein.

In conclusion, we have developed the first highly efficient and widely applicable method for cysteine-selective bioconjugation of phosphane ligands. Also a protein/phosphane/rhodium complex was successfully synthesized. Whereas Ward and others demonstrated the power of combining phosphane/transition-metal catalysts with proteins, this development holds great promise for the use of a wide range of protein structures as templates for the preparation of phosphane bearing artificial metalloenzymes.

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