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# Non-covalent binding of a transmetalated Ni-bacteriochlorophyllide *a* to four-helix bundle proteins obtained by orthogonal combinatorial synthesis

# H. Snigula<sup>1</sup>, B. Monien<sup>2</sup>, F. Drepper<sup>2</sup>, N. de Jonge<sup>2</sup>, W. Haehnel<sup>2</sup>, <u>H. Scheer<sup>1</sup></u>

 <sup>1</sup>Botanisches Institut, Universität, Menzinger Str. 67, D-80638 München, DE, Tel: +49-89-17861-295, Fax: +49-89-17861-185, email: scheer-h@botanik.biologie.uni-muenchen.de
<sup>2</sup>Institut für Biologie II / Biochemie, Universität, Schänzlestraße 1, D-79104 Freiburg, DE, Tel: +49-761-203-2690, Fax:+49-761-203-2601, email: haehnel@ruf.uni-freiburg.de

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#### Introduction

Cyclic tetrapyrroles are ubiquitous cofactors of biological catalysis, involved in a multitude of reactions. The chemical and spectroscopic properties of cyclic tetrapyrroles are tuned to a considerable degree by interactions with the native environment. In heme proteins, these are mainly neighboring the amino acid residues of the binding proteins. In chlorophyll proteins, there are in addition frequently strong interactions with neighboring chromophores.

Based on a wealth of structural information, tetrapyrrole proteins have been early targets to critically test our current knowledge on protein design. Degrado et al. have shown, that *bis*-helical synthetic proteins can dimerize under the influence of heme to form a four-helix bundle which binds one or two tetrapyrroles in its interior (Rabanal et al. 1995), and which can further aggregate to higher-order structures. (Robertson et al. 1994). Using an orthogonal design, four helices have been grafted on a cyclic peptide, to which natural and synthetic cofactors were then attached (Rau et al. 2001, Rau & Haehnel 1996, Katz et al. 1998)). By exploiting the second face of the cyclic matrix peptide, it was furthermore possible to covalently attach the construct onto a gold electrode and transfer electrons between the cofactors and the metal (Katz et al. 1998). More recently, metal centers have been created binding (Schnepf et al. 2001).

A particular advantage of the orthogonal systhesis is, that relatively large proteins (>12 kDa) can be constructed in a controlled, modular fashion from individual short (30 aa) helices and a differentially de-protectable cyclic peptide. Three different helices can be attached by conventional peptide chemistry a defined sequence and orientation to the four cysteins, and recent progress in protective group chemistry has increased this number to four. The sytem can be further exploited by parallel syntheses on solid supports (Rau et al. 2000).

The binding pockets of chlorophylls are more complex. This is due to the chemical lability of the pigments, and to their structural complexity which includes a number of functional peripheral substituents, the extra isocyclic ring E, the partial hydrogenation at rings D (and in bacteriochlorophylls (BChl) also ring B), and the long hydrophobic esterifying alcohol at C- $17^3$ . Only few synthetic chlorophyll-binding proteins have therefore been investigated. Besides a number of truncated or otherwise modified natural proteins ((Meadows et al. 1995), (Paulsen & Kuttkat 1993)), two peptides have been shown to interact with Chl in solution ((Miyake et al. 1998)). In a first attempt to design a total-synthetic Chl protein (Rau et al. 2001), we have recently chosen to (i) remove the phytyl side chain which renders the pigment very hydrophobic and which is unlikely to fit into a four-helix bundle, and (ii) to covalently bind the pigment *via* C=O groups to the protein carrying a hydroxylamino side chain near the position of the central His, which is placed appropriately to bind the central Mg of the (bacterio)chlorophyll. The covalent binding allows for a tighter control of the attachment, but

the approach is somewhat unsatisfactory. We now report non-covalent binding of the BChl derivative, [Ni]-BChl, to a combinatorial library of orthogonal four-helix bundle proteins.

### **Experimental**

Bacteriopheophytin *a* was metalated with Ni *via* the Cd-complex to Ni-BPhe (Hartwich et al. 1998), and the phytyl-ester cleaved with trifluoroacetic acid to yield Ni-Bpheid.

The synthesis of the helical peptides  $A_i$  (i=1-18) and  $B_j$  (j=1-13, Fig. 1) was performed with standard Fmoc protection as previously described, and sequences verified by mass spectrometry (Rau et al. 2000, Kolber et al. 2001).

The cyclic decapeptide-template was synthesized manually following standard Fmoc protocol (Rau et al. 2000, Rau et al. 1998) and cyclization after cleavage from the resin. It was then coupled onto derivatized cellulose membrane sheets (Rau et al. 2000). After drying under reduced pressure, the helices  $A_i$  were first coupled to the partially deprotected template, and after deprotection of the remaining two cys the helices  $B_j$  were attached as described before (Kolber et al. 2001). The syntheses were monitored by cleaving intermediates or products from test-spots and analyzing them by mass spectrometry.

Ni-BPheid was incorporated into the immobilized four-helix bundles by incubation of the cellulose membranes with a solution in Tris-HCl (100 mM, pH 7.5)/DMF (4:1, v/v). In order to test the stability of the four-helix bundle complexes with the pigments, the membranes were washed firstly with Na phosphate (100 mM, pH 7), then with Tris-HCl (100 mM, pH 7.5)/DMF (8:1, v/v), and finally with Tris-HCl (100 mM, pH 7.5)/DMF (4:1, v/v). After each washing procedure, the of UV-vis spectra (300 - 900 nm) of all spots were recorded using a microplate reader (Spectrafluor Plus, Tecan) combined with a fast diode array spectrometer (Tidas, J&M). It was possible to measure the spectra of all 48 spots on one membrane within 2 min, at 1 nm bandwidth and a S/N ratio sufficient for 2<sup>nd</sup> derivative spectroscopy.

#### **Results and discussion**

The variable amino acids of the two helices  $(X_i \text{ and } Z_j)$  were selected according to the following criteria: 1) the metal-chelating histidine on the binding helix (H-9) was maintained, and 2) the variable neighboring amino acids chosen to fill optimally the space around the bound metalloporphyrin. The second condition was tested in the following way: The metalloporphyrin was represented by a heme molecule, and the quality of packing was judged by letting the software (InsightII, MSI) fit water molecules in the free spaces around the heme.

The cellulose sheets with the protein library were soaked in buffer solutions containing Ni-BPheid, and subsequently washed with buffer containing increasing amounts of DMF. Most proteins bound considerable amounts of the pigment, while the background absorption was negligible. Visual inspection showed differences in total staining, differential loss of bound pigment during the washing procedures, and differences in the color of the bound pigment. The first indicates the amount of bound pigment, the second the stability of the binding, and the third the ligation status of its central metal. All three were then analyzed in detail by absorption spectroscopy of the spots. The Ni coordination state was analyzed by second derivative spectroscopy in the Q<sub>X</sub> region (530 – 630 nm). Four ligation states were defined, depending on the position of the Q<sub>X</sub> bands. These are four coordinate ( $n_c = 4$ ,  $\lambda_{max} = 544-551$  nm), five coordinate ( $n_c = 5$ ,  $\lambda_{max} = 583 - 590$  nm), as well as two six-coordinate states ( $n_c = 6$ ,  $\lambda_{max} = 608 - 613$  and 625 - 627 nm). The first three correspond to coordination states identified before by Noy et al. (2000). The two states with  $n_c = 6$  are rationalized by binding to only one of the His and to water in the case of  $\lambda_{max} \sim 610$  nm, and by binding to both His in the long-wavelength form.



Figure 1: Schematic representation of shielding helices A and binding helices  $B_j$ . The amino acids which were varied ( $X_n$  for  $A_i$  and  $Z_m$  for  $B_j$ ) are emphasized in gray. The tables contain the amino acids at these positions in the different helices synthesized.

The results can be summarized as follows:

- Ni-BPheid is well incorporated into the four helix bundles. The maximum optical density of the spots in the Q<sub>Y</sub> region was 0.93, which amounts to ~0.3 nmoles of pigment bound per spot, and even the weakest spot had still ≈0.1 nmoles, corresponding to 30% of this amount.
- 2) The staining (= binding) intensities correlated significantly with amino acid sequences of the binding helices  $B_j$ , with helices  $B_5$  and  $B_{12}$  binding particularly large amounts. These helices were also the most tightly binding ones, retaining up to 100% of the pigment bound after the third, as compared to the first wash. There is, however, also an obvious influence of the shielding helices  $A_i$ : binding to  $B_5$  is most effective with shielding helices  $A_7 A_{18}$ , with a peak at  $A_{14}/A_{15}$ . For helix  $B_{12}$ ; maximum binding is found with shielding helices  $A_5 A_{10}$ .
- 3) Ni-BPheid can be bound with coordination numbers  $n_c=4$ , 5 or 6, as inferred from the component analysis of the  $Q_X$  region (Noy et al. 2000). Strong binding is only observed with at least one extra ligand to the Ni. During washing, there is generally an increase in the relative amount of Ni-BPheid being bound in higher coordination states.
- 4) The coordination number is again correlated mainly with the amino acid sequence of the binding helices. For B<sub>3</sub>, B<sub>5</sub> and B<sub>11</sub>, the predominant species has  $n_c = 5$ , for B<sub>8</sub>, B<sub>10</sub> and B<sub>11</sub>, the predominant species has  $n_c = 6$ .
- 5) The ligation state also plays a role in the irreversible oxidation of the bacteriochlorin, Ni-BPheid, the corresponding chlorin, [3-acetyl]-Ni-Pheid.. It is most pronounced with n<sub>c</sub>=6.

6) After washing away the pigments with DMF, binding of other pigments can be tested using the same cellulose sheets. Some memory effects were observed after binding of the Fe tetrapyrrole (heme).

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