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Spectroscopic studies on peptides and proteins with cysteine-containing heme regulatory motifs (HRM)

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ABSTRACT

The role of heme as a cofactor in enzymatic reactions has been studied for a long time and in great detail. Recently it was discovered that heme can also serve as a signalling molecule in cells but so far only few examples of this regulation have been studied. In order to discover new potentially heme-regulated proteins, we screened protein sequence databases for bacterial proteins that contain sequence features like a Cysteine–Proline (CP) motif, which is known for its heme-binding propensity. Based on this search we synthesized a series of these potential heme regulatory motifs (HRMs). We used *cw* EPR spectroscopy to investigate whether these sequences do indeed bind to heme and if the spin state of heme is changed upon interaction with the peptides. The corresponding proteins of two potential HRMs, FeoB and GlpF, were expressed and purified and their interaction with heme was studied by *cw* EPR and UV–Visible (UV–Vis) spectroscopy.

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1. Introduction

Iron porphyrins (hemes) are found in most organisms in nature and are crucial components of many different biological processes, such as electron transport, gas transport and metabolism [1]. In the proteins exerting these functions, heme is usually tightly bound as a cofactor, and represents an integral part of the protein structure. The heme molecule is able to harbour two oxidation states of iron (Fe(II) and Fe(III)). If the 6th coordination site is not occupied or occupied by a weak base the iron adopts the high-spin state ($S = 2$ for Fe(II) and $S = 5/2$ for Fe(III)) whereas upon coordination by a strong base the low-spin state ($S = 0$ for Fe(II) and $S = 1/2$ for Fe(III)) state is attained. This property is central to the function of many of the aforementioned proteins.

More recently, it was found that instead of acting as a fixed prosthetic group, heme can also act as a signalling molecule by temporarily binding to a protein and thereby regulating its conformation and/or function. Well-studied examples are the heme-dependent regulation of gating in the human big potassium (BK) channel Slo1 [2–5] and the iron response regulator (Irr) [6,7]. Another example is the nuclear hormone receptor Rev-erbβ, where the Fe(III)-heme is either five- or six-fold coordinated depending on the redox conditions [8].

These findings raised the question whether heme-based regulation is a widespread phenomenon in nature [3,9]. As a possible way to investigate this question, different peptide libraries were synthesized and screened for heme-binding properties [4,10]. In particular, a combinatorial peptide library revealed useful information about the influence of the coordinating amino acid and the flanking regions on the heme-binding properties [4]. The results of these efforts were compiled into lists of short sequence motifs, so-called potential "heme regulatory motifs" (HRMs). The HRMs were further grouped based on features in their amino acid sequence [4,11,12]. The so far best-studied HRM class contains the "CP motif", i.e. the iron coordinating amino acid cysteine followed by a proline residue. Recently, the molecular structures of different peptides harbouring such a CP-motif were solved by NMR spectroscopy [11,12]. The structures revealed that the unique properties of the proline side chain lead to a distinct conformation of the sequence C-terminal of the cysteine, i.e. a kink induced in this region directs the C-terminus away from the protoporphyrin ring. This enables the cysteine to strongly coordinate heme via its exposed thiol group. Other HRMs contain the amino acids tyrosine or histidine as coordinating amino acids [4]. Using UV–Vis spectroscopy, it was possible to derive binding constants of the peptide:heme interaction and to define subclasses for each motif, which are based on the spectral changes in the Soret region of the heme spectrum [4,11,12]. This detailed knowledge about HRMs can now be used to screen sequence databases for proteins containing HRM and to investigate whether a particular protein is indeed regulated by heme [4,11].

As mentioned above, the electronic and magnetic properties of the heme iron are an important factor in the function of many well-studied hemoproteins. This leads to the question, whether this is also

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true for the regulatory role of heme, i.e. if a given HRM induces a certain spin state in the heme molecule. The aim of this work was to investigate this by analysing a small set of peptides derived from: i) a combinatorial peptide library (high throughput screening—HTS) [4] and ii) proteins with known HRMs [4,11,12]. Peptides that bound heme in the HTS were then screened against protein sequence databases to find new potentially heme regulated proteins. Continuous wave (*cw*) EPR spectroscopy was employed to measure the heme–peptide interaction and to find out the spin state of the bound heme. In addition, two proteins containing a predicted HRM, namely ferrous iron transport protein B (FeoB) [13,14] and glycerol uptake facilitator protein (GlpF) [15] were expressed and purified in order to investigate with EPR and UV–Vis whether they indeed interact with heme.

2. Experimental

The peptide synthesis and purification were performed as described earlier [13,14].

2.1. Cloning, expression and purification of $NFeoB^{1–273}$

The *feoB* gene from *Escherichia coli* BL21 was cloned into pBADHisMBPTEV (gift from Huanting Liu, University of St Andrews) and a STOP codon was inserted at position 274. Mutant C143A was prepared using PCR techniques [16]. The plasmid was transformed into *E. coli* C43 cells and typically 3 l of cells were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin with shaking (180 rpm). At an OD of ~0.5, protein expression was induced by adding L-arabinose at a final concentration of 0.2%. The expression was allowed to proceed for 4 h at 37 °C with shaking (180 rpm). Cells were then harvested and the pellet resuspended in buffer A (50 mM Tris–Cl pH 8.0, 50 mM NaCl). A cell disrupter (Constant Systems) was used to disrupt the cells. Cell debris and insoluble material were separated by centrifugation at 20,000 rpm in a Beckman JA 25.50 rotor. The supernatant was then loaded onto a HisTrap FF column (5 ml) using buffer A, washed and eluted with a gradient running from 0 to 100% buffer B (buffer A + 1 M imidazole). The protein was then loaded onto a MonoQ 5/50 column using buffer A and eluted with a gradient running from 0 to 100% buffer C (buffer A + 1 M NaCl). 4 mg TEV (tobacco etch virus) protease was added to the sample to cleave the His_6-tag. The sample was then again loaded onto a HisTrap FF column (5 ml). This time the flowthrough was collected, while uncleaved protein and TEV protease bound to the column material. To remove any remaining traces of uncleaved NFeoB, the protein was then loaded onto an amylose affinity column (NEB) equilibrated with buffer D (20 mM Tris–Cl pH 8.0, 150 mM NaCl). The flowthrough was concentrated to ~2 ml and loaded onto a Superdex 200 16/60 column using buffer D. The protein eluted at ~90 ml, corresponding to monomeric $NFeoB^{1–273}$. Fractions from the gel filtration peak were collected, concentrated to ~60 mg/ml and frozen in 50 μl aliquots. The yield was ~10 mg/l culture. The binding assays were performed with freshly prepared protein. A western blot with anti-His antibody revealed that our purification protocol indeed removed all traces of the N-terminal His_6 tag (Supporting Fig. S4).

2.2. Cloning, expression and purification of GlpF

The *glpF* gene from *E. coli* BL21 was cloned into pBADHisTEV (gift from Huanting Liu, University of St Andrews). The plasmid was transformed into *E. coli* C43 cells and typically 3 l of cells were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin with shaking (180 rpm). At an OD of ~0.5, protein expression was induced by adding L-arabinose at a final concentration of 0.2%. The expression was allowed to proceed o/n at 37 °C with shaking (180 rpm). Cells were then harvested and the pellet was resuspended in buffer A (50 mM Tris–Cl pH 8.0, 50 mM NaCl). A cell disrupter (Constant Systems) was used to disrupt the cells. Cell debris and insoluble material were separated by centrifugation at 10,000 rpm in a Beckman JA 25.50 rotor. The supernatant was then centrifuged again at 75,000 ×*g* for 75 min and the membrane pellet was resuspended in extraction buffer (buffer A + 1% dodecylmatoside (DDM)). The extraction was allowed to proceed o/n at RT. The supernatant was then loaded onto Ni-agarose beads using buffer C (buffer A + 0.024% DDM + 20 mM imidazole) and incubated for 1 h. The beads were washed and the protein eluted with elution buffer (buffer C + 1 M imidazole). The elution was diluted to 50 ml with buffer C (w/o imidazole) and supplemented with 4 mg TEV protease, 1 mM TCEP and 0.5 mM EDTA. The following day, the sample was again loaded onto the Ni-beads. This time the flowthrough was collected, while uncleaved protein and TEV protease bound to the column material. The flowthrough was concentrated to ~2 ml and loaded onto a Superdex 200 16/60 column using gelfiltration buffer (20 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.024% DDM). The protein eluted at ~60 ml, corresponding to tetrameric GlpF. Fractions from the gelfiltration peak were collected, concentrated to ~10 mg/ml and frozen in 50 μl aliquots. The yield was 1–2 mg/l culture.

2.3. EPR spectroscopy

Hemin chloride (heme) was purchased from Carl Roth. Fe(III) heme and the peptides were mixed in a 1:1 ratio in phosphate buffer (100 mM phosphate buffer with 50 mM NaCl, pH 7.0) to yield a final concentration of 300 μM. The complexes were incubated for 30 min in the dark and centrifuged prior to filling into EPR sample tubes. Hemin chloride was dissolved in 30 mM NaOH and diluted with buffer to yield a final concentration of 600 μM with pH 7.0. Fe(III) heme solution, 10-times (10×) concentrated buffer, water and GlpF were mixed, incubated in the dark for 1 h, filled into EPR sample tubes. The heme/GlpF ratio has been 1:1 with a final heme concentration of 200 μM. Fe(III) heme solution, buffer (0.1 M HEPES, pH 7.0) and $NFeoB^{1–273}$ were mixed, incubated in the dark for 1 h, filled into EPR sample tubes. The heme/$NFeoB^{1–273}$ ratios have been 1:1 and 1:4 with a final heme concentration of 200 μM. In reference samples the volume of the protein solutions were substituted with buffer—in the text referred to as free heme solution. Prepared samples were frozen and stored in liquid nitrogen. EPR spectra were recorded on an ELEXSYSII E580 X-band EPR spectrometer from Bruker equipped with a Super High Sensitivity (SHQ) resonator for the X-band measurements. The temperature was controlled with an Oxford ESR900 cryostat in combination with an ITC503S. The samples were measured at 10 K with a modulation amplitude of 5 G, a time constant of 20.48 ms, a conversion time of 21 ms and in a field range between 0 G and 4000 G with 1 point per G resolution. The spectra were background corrected.

2.4. UV–Vis spectroscopy

The peptides were measured and analysed as described earlier [4,11,12]. For the proteins, a dilution series of each protein was prepared with protein concentrations ranging from 500 μM to 0.02 μM, 0 μM (control). The protein was diluted in binding buffer (0.1 M HEPES, pH 7.5). For each concentration, a binding reaction was setup by adding 10 μl heme (500 μM), 10 μl 10× binding buffer to the protein dilution. The reactions were left to incubate at room temperature for 1 h. For each concentration, three individual samples (3 μl) were taken and a UV–Vis spectrum was recorded on a Thermo Scientific NanoDrop 2000 spectrometer. The three spectra of each concentration were averaged before further processing. To correct the background scattering, a straight line was fit to the spectrum between 460 and 650 nm. Fitting with a $1/\lambda^{-4}$ function was not possible due to the crowded spectra in the UV area. The line was then subtracted from each spectrum (Figure S-3C). To analyse the heme–protein interaction, the ΔA374 was recorded and plotted against the protein concentration. The whole procedure, including preparation of the dilution series, was repeated three times. To calculate K_D values, a Langmuir equation ($y = a \times [FeoB] / (b + [FeoB]) + offset$)

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