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Engineering redox functions in a nucleic acid binding protein

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A nucleic acid binding protein, rop, has conserved topology with a number of redox proteins; this is exploited to engineer haem binding, expanding its function as a redox protein.

Engineered protein structures with specific activities on a par with their natural counterpart, but without the associated cellular functions are an attractive goal for chemistry. This has been addressed either by the *de novo* synthesis of peptides using simplified templates[1–3] or by mutating wild-type redox proteins,[4] or more recently by modular assembly of existing proteins.[5] An alternative approach, highlighted here, is to take an existing simple and stable protein fold as a structure in which to insert new functions. This approach mimics the natural process of protein evolution, where a diversity of function can be found within a protein structural superfamily.[6]

Evolutionary links are masked by diversity in sequence but can now be revealed by close examination of the expanding database of protein structures. This allows the identification of conserved protein folds, sometimes suggesting unsuspected functional properties. In this study, the structure of the *E. coli* repressor of primer (rop), a regulatory protein that binds RNA,[7] was selected as a target to search for structural neighbours with diverse function. Rop is a homo-dimeric protein that forms a very stable and well characterised four-helix bundle.[8,9] A monomeric derivative of rop has been engineered so that the four helices are expressed as a single polypeptide chain, with advantages for the introduction of mutations in unique positions.[10,11]

The demonstration of electron transfer reactions in DNA[12,13] together with the regulatory function found for cytochrome c over cellular events such as apoptosis suggests a link between these traditionally separate functions.[14,15] The relationship between the rop structure and that of natural redox proteins was investigated using the FSSP (Fold classification based on Structure–Structure alignment of Proteins),[16] and the Vector Alignment Search Tool (VAST) of the National Center for Biotechnology Information.[17] Both are algorithms designed to search for neighbours of a given structure. For FSSP the target was the experimental structure for native, dimeric rop[8] and for VAST (that requires a target structure to have more than two secondary structure elements) a model for the monomeric rop structure. This was generated by homology modeling using the program Insight II (MSI, Biosym) using the NMR structure of the dimeric form as a template. Both algorithms returned high scoring matches for redox proteins, these are shown in Table 1.

The proteins identified range from four-helix bundle proteins, for example cytochrome b562, cytochrome c′ and myohemerythrin, to proteins that contain a rop-like fold as a domain or as part of a domain, for example cytochrome c oxidase and the photosynthetic reaction center. The structural similarity between rop and, for example, cytochrome c′ becomes apparent in the superposition of the two structures (Fig. 1A). This similarity is quantified in a low VAST p-value of 0.008, a probability score that indicates that the match is very unlikely to have occurred by chance simply because the four-helix bundle is a common motif.

On the basis of this evidence protein engineering was used to study whether the nucleic acid binding rop can be converted into a redox counterpart by incorporating a haem-binding site.

Monomeric rop provides a better template for engineering than its dimeric precursor because of the ability to introduce single mutations. The initial step was to remove potential non-specific ligands by the replacement of endogenous histidine

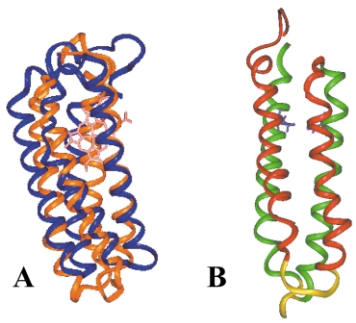

Fig. 1 A. Cα-backbone of the monomeric rop model (orange) superimposed to that of cytochrome c' (blue). The alignment was generated with VAST[17] and the image with *InsightII* (MSI). **B.** Model of monomeric rop showing the engineered histidine residues L56H and L113H (blue).

Table 1 Close redox protein structural neighbours of the rop protein structure identified by comparison algorithms‡

			FSSP[16]		VAST[17]	
Protein chain	PDB ID	Chain	Z Score[a]	RMSD[b]	Score[c]	P-VAL[d]
Cytochrome c oxidase	2OCC	C (5)	5.6	2.6	6.4	0.0018
Myohemerythrin	2MHR	—	4.0	2.2	6.3	0.0004
Cytochrome b_{562}	256B	A	4.7	2.6	6.2	0.0023
Cytochrome c′	1CPO	—	5.0	2.2	6.1	0.0008
Photosynthetic reaction centre	6PRC	L	4.7	2.7	—	—
Rubrerythrin	1RYT	—	4.0	2.0	—	—
Ferritin	1GGH	A	4.7	1.5	—	—

[a] Dimeric rop was the target for the FSSP structure comparison algorithm. Statistical significance of the best domain–domain alignment. [b] Root-mean-square deviation of C-α atoms in the superposition. [c] Monomeric rop was used as the target structure for the VAST structure comparison algorithm. The VAST structure similarity score relates the quality and number of secondary structure superpositions. [d] A measure of the significance of the comparison expressed as a probability.

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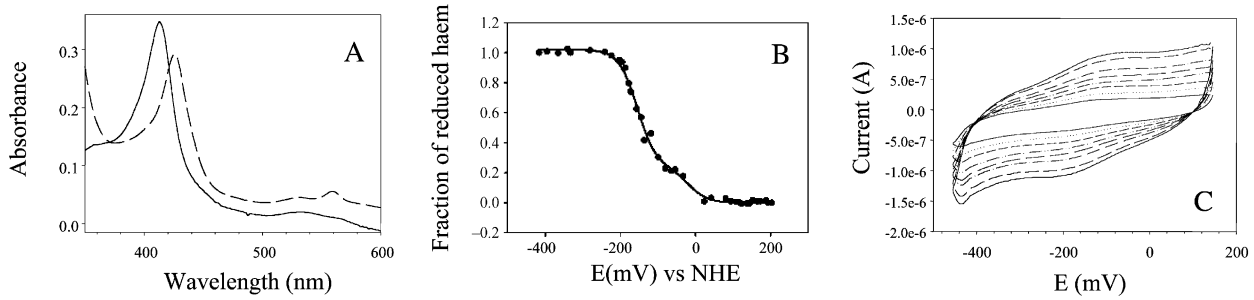

Fig. 2 Spectroscopic and electrochemical properties of the rop heme-bound protein.† **A.** Visible spectra of oxidized and reduced form. **B.** Spectrophotometric titration with fitting to a sum of two one-electron Nernst equation. **C.** Cyclic voltammogram. Potentials (*E*) are expressed relative to normal hydrogen electrode.

residues creating the mutations H76A, H78W, H107A, H109W. The putative haem binding site was introduced by engineering the further mutations L56H, L113H, (Fig. 1B). This haem-binding variant (rop-56H/113H) was heterologously expressed from the pMR103 plasmid[18] in *E. coli* BL21 (λDE3) and purified to homogeneity. Haem was incorporated into the apo-protein by addition of a 4-fold molar excess of hemin chloride and removal of free haem by anion exchange chromatography. Circular dichroism confirmed that the mutants are virtually identical to the wild type. The resulting haem binding rop protein has spectral properties characteristic of native b-type cytochromes, Fig. 2A. The oxidized protein has a λ_{max} at 413 nm, that upon reduction with sodium dithionite shifts to 426 nm with distinct α and β peaks at 559 nm and 531 nm respectively. These spectral characteristics are reminiscent of b-type cytochromes with a six coordinate low spin iron,[19] and suggests that the haem group has been incorporated into the helix bundle.

The electrochemical properties of this novel haem-protein, determined by spectroelectrochemistry and cyclic voltammetry,† are presented in Fig. 2. The redox titrations with sodium dithionite (Fig. 2B) show one larger component (78%) at −154 ± 2 mV and one smaller (22%) at 17 ± 9 mV, relative to the normal hydrogen electrode. The presence of two reduction potentials is likely to be due to two non-equivalent orientations of the haem in the engineered rop. This is at present being investigated. Only one mid point potential of $E_m = -134 \pm 13$ mV could be measured using cyclic voltammetry on a bare glassy-carbon electrode using a modified Hagen cell[20] (Fig. 2C).

These reduction potentials are consistent with those measured for natural cytochromes and haem-containing synthetic peptides, that cover the range of −475 to +310 mV.[21–24] The properties of the rop haem binding protein are at present being optimised with the creation of a cavity that accommodates the haem macrocycle with a unique orientation, with the possibility of tuning the redox potential. It has been demonstrated that the redox potential of cytochrome b562 can be varied by over 100 mV by changing the amino acids in the vicinity of the haem[19] and Dutton and coworkers demonstrated that by utilizing alternative porphyrins it was possible to modulate the redox potential of their designed haem protein by as much as 225 mV.[25] It is anticipated that the redox properties of this designed protein could likewise be 'tuned' as required for particular applications.

In conclusion, this work demonstrates that existing natural protein scaffolds can be used to create novel proteins with a specified function. This utilizes principles found in nature and allows advantage to be taken of high levels of expression and stability of natural proteins. The four-helix-bundle motif has been adapted to bind other small co-factors, for example diiron[26] and such adaptations should also be possible for monomeric rop. It is anticipated that engineered proteins based on natural proteins will be of use both as tools for the study of physiochemical properties or as the biological basis of novel devices for nanobiotechnology.

Notes and references

† For FSSP http://www.ebi.ac.uk/dali/fssp and VAST http://www.ncbi.nlm.nih.gov/Structure/

‡ Redox titrations: 40 μM protein in 10 mM sodium phosphate, pH 7.8, 100 mM sodium chloride, phenazine methosulfate (5 μM), duraquinone (5 μM), 2-hydroxy-1,4-napthoquinone (5 μM), benzyl viologen (2 μM), indigo carmine (0.5 μM) and resofurin (0.5 μM). Cyclic voltammetry: 20 μM protein in 10 mM phosphate pH 7.8, 100 mM sodium chloride.

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