Role of the cysteine-rich region of the iron-responsive GATA factor Fep1 in [2Fe-2S] cluster binding

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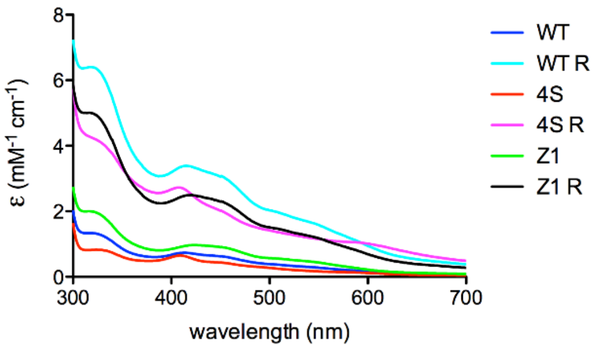
Introduction

Fep1 is a member of the family of fungal iron-responsive GATA-type transcriptional repressors. The N-terminal domain of these proteins is characterized by the presence of two zinc fingers of the Cys_2-Cys_2 type and a set of four highly conserved cysteines arranged in a Cys-X_5-Cys-X_8-Cys-X_2-Cys motif located between the two zinc fingers. By using spectroscopic techniques (Raman, UV/VIS Electronic Absorption, SAXS), we have shown that Fep1 of the methylotrophic yeast *Pichia pastoris* is a dimer able to bind iron in the form of a [2Fe-2S] cluster (1). The aerobically purified protein appears to be a mixture of two forms: one where the [2Fe-2S] cluster is bound by the central cysteines (Cys102-Cys108-Cys117-Cys120) and one where the cluster ligands are Cys65-Cys68, belonging to ZnF1. When the four central cysteines are replaced by serines (this mutant is hereafter called 4S) the [2Fe-2S] cluster is bound by Cys65-Cys68 within ZnF1; conversely, when cysteines of ZnF1 are changed to serines (mutant Z1) the cluster can only be ligated by the central cysteines. The two clusters possess full cysteinyl ligation and have clearly distinguishable electronic absorption and resonance Raman spectroscopic signatures (1).

In this work, we extend the characterization of Fep1 by analysis of the optical and CD spectroscopic properties of a set of mutants targeting the cysteine residues belonging to the strictly conserved Cys-X_5-Cys-X_8-Cys-X_2-Cys motif, in order to evaluate their role as [2Fe-2S] ligands. These mutations have been introduced in a Z1 background to prevent binding of the [2Fe-2S] cluster to Cys65-Cys68 of ZnF1.



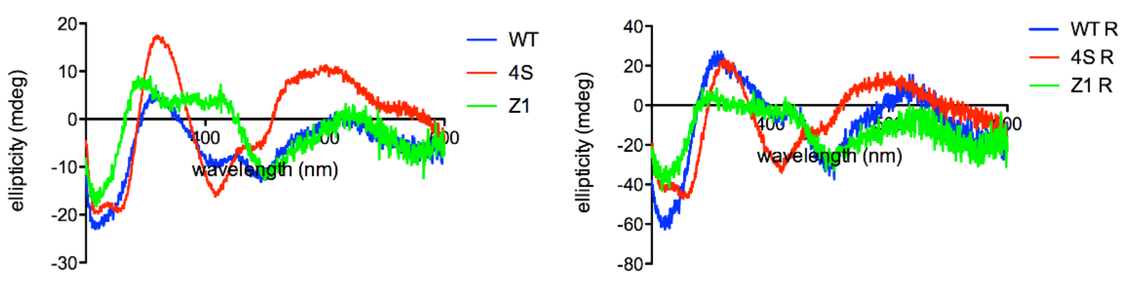


***Figure 2*. Reconstitution of Fep1**. Absorption and CD spectra of Fep1 WT and cysteine mutants 4S and Z1 before and after aerobic reconstitution with stoichiometric $FeCl_3$ and Na_2S in the presence of TCEP 1 mM.

Iron and acid-labile sulfur content of Fep1 WT and cysteine mutants

Fep1	Fe/protein	S/protein	Fe/protein	S/protein
	As-purified		After reconstitution	
WT	0.13	0.23	0.99	0.70
4S	0.10	0.10	1.07	0.38
Z1	0.18	0.28	0.84	0.75
Z1-4S	0.03	0.17		
Z1-C12S	0.11	0.34	0.35	0.59
Z1-C34S	0.04	0.29	0.45	0.56
Z1-C13S	0.04	0.19	0.52	0.43
Z1-C14S	0.02	0.23	0.47	0.33
Z1-C23S	0.05	0.23	0.41	0.44
Z1-C24S	0.02	0.22	0.52	0.42

Expression and purification of Fep1 in E. coli

The DNA-binding domain of *P. pastoris* Fep1 (amino acid residues 1-208) with a Flag tag at the N-terminus was cloned in pET28a and expressed in *E. coli* BL21(DE3). Cells were induced with 0.1 mM IPTG for 4 h at 37°C in LB medium supplemented with $ZnSO_4$ 50 μM and $Fe(NH_4)SO_4$ 20 μM.

Cells were disrupted by sonication and Fep1 WT and all mutants were purified on a CM-Sephadex C-50 column equilibrated in 25 mM MOPS pH 7 containing 30 mM NaCl. After washing with 10 column volumes of equilibration buffer followed by 3 column volumes of MOPS buffer containing 90 mM NaCl, Fep1 was eluted with MOPS buffer containing 250 mM NaCl. All purification steps were carried out aerobically.

	ZnF1				ZnF2	
	C44-C47	C65-C68	C102-C108	C117-C120	C164-C167	C185-C188
WT	C C	C C	C C	C C	C C	C C
4S	C C	C C	S S	S S	C C	C C
Z1	C C	A A	C C	C C	C C	C C
Z1-4S	C C	A A	S S	S S	C C	C C
Z1-C12S	C C	A A	S S	C C	C C	C C
Z1-C34S	C C	A A	C C	S S	C C	C C
Z1-C13S	C C	A A	S C	S C	C C	C C
Z1-C14S	C C	A A	S C	C S	C C	C C
Z1-C23S	C C	A A	C S	S C	C C	C C
Z1-C24S	C C	A A	C S	C S	C C	C C

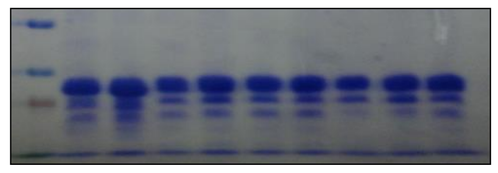


***Figure 1*. Scheme of Fep1 mutants and SDS-PAGE analysis of purified proteins**. The red MW marker band corresponds to MW 20 kDa).

Spectroscopic characterization of Fep1

Electronic absorption and circular dichroism (CD) spectroscopic analyses of Fep1 WT, 4S and Z1 before and after reconstitution by aerobic addition of $FeCl_3$ and Na_2S in the presence of TCEP showed a substantially increased intensity of the optical and CD spectra, indicating that the iron cluster could be reconstituted in these conditions (Fig. 2). Iron stoichiometry after reconstitution was close to 1 Fe/protein monomer, acid-labile sulfur also increased to about 1 S/protein monomer. Fep1 Z1 cysteine mutants showed an increase in the intensity of the optical spectra, however the CD spectra remained essentially featureless (Fig. 3). Iron and acid-labile sulfur content after reconstitution was about 0.35-0.5 atoms/protein.

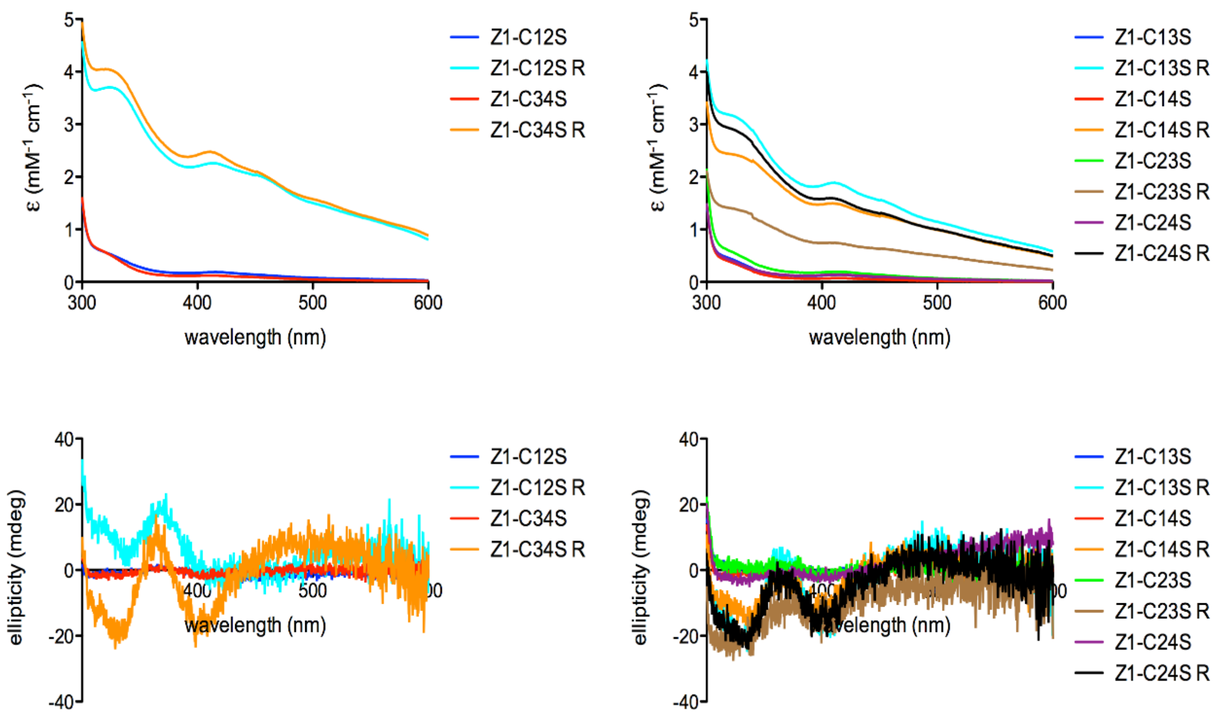


***Figure 3*. Optical and CD spectra of Fep1 cysteine mutants**. Absorption and CD spectra of Fep1 cysteine mutants before and after aerobic reconstitution with stoichiometric $FeCl_3$ and Na_2S in the presence of TCEP 1 mM.

CONCLUSIONS

Iron sensing by the transcriptional repressor Fep1 from *P. pastoris* is exerted through its ability to bind a [2Fe-2S] cluster. The results obtained by analysis of a set of cysteine mutants targeting the strictly conserved Cys-X_5-Cys-X_8-Cys-X_2-Cys motif suggest that all four cysteine residues are essential because replacing them with serines in different combinations invariably produces a protein unable to correctly bind the [2Fe-2S] cluster.

References

1. Cutone A, Howes BD, Miele AE, Miele R, Giorgi A, Battistoni A, Smulevich G, Musci G, Bonaccorsi di Patti MC. (2016) *Pichia pastoris* Fep1 is a [2Fe-2S] protein with a Zn finger that displays an unusual oxygen-dependent role in cluster binding. *Sci Rep*. 6, 31872.