Identification and characterization of a eukaryotically encoded rubredoxin in a cryptomonad alga[1]

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Abstract We have identified an open reading frame with homology to prokaryotic rubredoxins (rds) on a nucleomorph chromosome of the cryptomonad alga *Guillardia theta*. cDNA analysis let us propose that the rd preprotein has an NH_2-terminal extension that functions as a transit peptide for import into the plastid. Compared to rds found in non-photosynthetic prokaryotes or found in bacteria that exhibit an anoxigenic photosynthesis apparatus, nucleomorph rd has a COOH-terminal extension, which shows high homology exclusively to the COOH-termini of cyanobacterial rds as well as to a hypothetical rd in the *Arabidopsis* genome. This extension can be divided into a putative membrane anchor and a stretch of about 20 amino acids with unknown function linking the common rd fold to this anchor. Overexpression of nucleomorph rd in *Escherichia coli* using a T7 RNA polymerase/promotor system resulted in a mixture of iron-containing holorubredoxin and zinc-substituted protein. Preliminary spectroscopic studies of the iron form of nucleomorph rd suggest the existence of a native rd-type iron site. One-dimensional nuclear magnetic resonance spectroscopy of recombinant Zn-rd suggests the presence of a stable tertiary fold similar to that of other rd structures determined previously.

Key words: Rubredoxin; Cryptomonad; Nucleomorph; Transit peptide; Iron binding site

1. Introduction

Rubredoxin (rd) is a small non-heme–iron protein (about 6 kDa), characterized as an electron carrier in prokaryotes. Rd contains a single iron atom, tetrahedrally ligated by four cysteines but no inorganic sulfur. The first rd gene was isolated from *Clostridium pasteurianum* [1], and most of the to date sequence data base entries are from anaerobic eubacteria. It is believed that only a minority of the different metabolic pathways in which rd is involved are characterized. For example, participation of rd as a cofactor of a terminal oxidase is described in the sulfate reducing eubacterium *Desulfovibrio gigas* [2] and in hydrogen oxidation from *Azotobacter vinelandii* [3].

Rd is also present in aerobic bacteria. *Acinetobacter calcoaceticus* for example harbors rd, characterized as a cofactor in alkane degradation [4]. In *Pseudomonas oleovorans*, a polypeptide about twice the size of the typical rd has been identified and was shown to be involved in hydroxylation of alkanes and fatty acids [5,6]. Recently, a rd gene encoding a unique COOH-terminal extension was identified in the genome sequence of the cyanobacterium *Synechocystis* sp. strain PCC6803 [7].

Guillardia theta is a representative of the cryptomonads, which are unicellular biflagellate algal cells. In addition to the common three DNA-containing organelles in photosynthetic eukaryotes, a fourth one, the so-called nucleomorph, was identified in these organisms [8]. Nucleomorphs harbor very small eukaryotic genomes (520–660 kb) organized in three tiny chromosomes [9]. Phylogenetic analysis has shown that nucleomorphs are remnant nuclei of former free-living algae that have been engulfed by another eukaryotic cell and established as a phototrophic symbiont [10,11]. Here, we report evidence of a eukaryotic rd by analyzing a nucleomorph-encoded protein localized on chromosome II of the vestigal nucleus of the cryptomonad *G. theta* secondary symbiont.

2. Materials and methods

2.1. Strains and media

G. theta was cultivated as described previously by Douglas [12].

Escherichia coli strain BL21(DE3) [13] was used for expression of nucleomorph rd. TB growth medium and M9 minimal medium [14] containing 2 mM $MgSO_4$, 0.01 mM Fe(III)-citrate, 0.1 mM $CaCl_2$, 0.4% glucose and 2 ml/l TS2 [15] were supplemented with 200 μg/ml ampicillin.

2.2. Cloning strategy

DNA of nucleomorph chromosome II was restricted with *Xba*I (Pharmacia, Freiburg, Germany) and cloned into pBluescript plasmid vector (Stratagene, La Jolla, CA, USA) [16]. Automated sequencing of plasmids was done on an ALF Express (Pharmacia, Freiburg, Germany) using the Thermo-Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-GTP (Amersham, Freiburg, Germany).

The cDNA library of *G. theta* [17] was screened according to the manufacturer's (Stratagene, La Jolla, CA, USA) protocols.

2.3. Construction of the expression vector

With the primers NMR5rub (ctg aaa aat cca tgg aga tcg acg aag) and NMR3rub (gaa tgc tca gct taa cca tat ttt tg), a DNA fragment spanning amino acids 57–126 of the nucleomorph rd was generated by PCR [26]. The amplificate was restricted with *Nco*I and *Bpu*1102I and

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Abbreviations: DEAE, diethylaminoethyl; ε, extinction coefficient; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI, matrix-assisted laser desorption ionization; NMR, nuclear magnetic resonance; OD_{600}, optical density measured at 600 nm; PAGE, polyacrylamide gel electrophoresis; rd, rubredoxin; Tris, tris(hydroxymethyl)aminoethane

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cloned into a likewise digested pET28a (Novagen, Madison, WI, USA) resulting in the expression vector pRUB. The nucleotide sequence of the pRUB insert was verified by sequencing as described above.

2.4. Purification of heterologous rd

Transformed *E. coli* BL21(DE3) with pRUB were cultured overnight in 50 ml TB/ampicillin at 37°C. A fermenter (Typ L1523 Bio-Engineering, Switzerland) containing 7 l TB medium with ampicillin was inoculated to optical density measured at 600 nm (OD_{600}) = 0.1. For growth in minimal medium, six 1 l flasks containing 500 ml medium were inoculated to the same OD. After the OD_{600} reached 0.8, the T7 RNA polymerase was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM. Cells were collected by centrifugation (15 min, 6000 × *g*) 4 h after induction. The cell pellet was resuspended in 10 mM tris(hydroxymethyl)aminoethane (Tris)–HCl (pH 8.0), 0.1 mM PMSF, 20 μg/ml lysozyme and 20 μg/ml DNase I and incubated on ice for 30 min. Bacteria were sonicated using a Labsonic U (Braun, Melsungen, Germany) sonicator at 8000 Hz, 200 W for 5 min on ice. The lysate was centrifuged at 30 000 × *g* for 2 h at 4°C, and the light red supernatant was dialyzed against water for 16 h at 4°C. All subsequent chromatography steps were done at 4°C. The red dialysate was loaded onto a XK26/20 column filled with 60 ml diethylaminoethyl (DEAE) Sepharose CL-6B (Pharmacia, Freiburg, Germany) equilibrated with 10 mM Tris–HCl pH 8.0 at a flow rate of 1.5 ml/min. The column was washed with 300 ml Tris–HCl pH 8.0, and bound protein was eluted with a linear gradient of 0–300 mM NaCl in 70 min. The colored fractions were combined, dialyzed against distilled water for 16 h (Spectra/Por membrane, MWCO: 1000) at 4°C and lyophilized (Christ Alpha 1-4, Osterode, Germany). The concentrated protein was resuspended in 10 mM Tris–HCl, pH 8.0, 200 mM NaCl and loaded onto a Superdex-HiLoad 26/60 gel filtration column (Pharmacia, Freiburg, Germany) at a flow rate of 1.5 ml/min. The eluent was 10 mM Tris–HCl, pH 8.0. Colored fractions were collected, and the purity of nucleomorph rd was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Fractions with less then 5% of foreign proteins estimated from Coomassie blue-stained protein gels were dialyzed against distilled water for 16 h and concentrated by lyophilization. Iron and zinc forms of nucleomorph rd were resolved using a Mono-Q HR 10/10 column (Pharmacia, Freiburg, Germany). Up to 20 mg lyophilized protein was resuspended in 50 mM Tris–HCl, pH 8.5, loaded onto a Mono-Q column and eluted with a linear gradient of 0–300 mM NaCl within 60 min at a flow rate of 2 ml/min. The protein peaks were collected, dialyzed against distilled water and lyophilized.

Protein concentrations of samples containing oxidized iron as predominant metal were determined from absorbance at 490 nm using $\varepsilon_{490} = 8800\ M^{-1}\ cm^{-1}$ or from the absorbance at 280 nm using $\varepsilon_{280} = 17\,190\ M^{-1}\ cm^{-1}$, a value due to contributions of the iron cluster and as calculated with the ε_{280} value of *C. pasteurianum* rd as basis [18]. Concentrations of zinc-containing nucleomorph rd samples were determined by 280 nm absorbance using $\varepsilon_{280} = 5120\ M^{-1}\ cm^{-1}$, calculated from the Tyr content [19].

2.5. NH_2-terminal amino acid sequencing

The NH_2-terminal amino acids of nucleomorph rd were determined using standard Edman degradation methods [48].

2.6. Matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF-MS)

Mass spectroscopic analysis of the recombinant nucleomorph rd was performed on a Compact MALDI apparatus (Shimadzu, Japan) using sinapinic acid as matrix and bovine cytochrome *c* (Sigma, Deisenhofen, Germany) as standard. Nucleomorph rd apoprotein was obtained by dissolving lyophilized Zn-rd under acidic conditions (0.1 M HCl) and the solution was separated via a Mono-Q chromatography using the conditions given in Section 2.4.

2.7. Spectroscopic methods

UV/Vis: UV/visible absorption spectra were obtained on an Uvikon 930 spectrophotometer (Kontron-BIO-TEK, Neufahrn, Germany).

One-dimensional (1D) nuclear magnetic resonance (NMR): 1D NMR spectroscopy of Zn-rd was performed at 25°C, pH 6.5, in H_2O/D_2O (9:1, v/v) with 2 mM protein concentration. All spectra were recorded on a Bruker DRX600 spectrometer operating at a proton resonance frequency of 600 MHz, using a spectral width of 8389.3 Hz. The 1D spectra were acquired with excitation sculpting [20] for water suppression.

3. Results and discussion

3.1. Cloning and sequence analysis of the nucleomorph rd

Shotgun cloning and sequencing of the chromosomes of the remnant nucleus of the symbiont of the cryptomonad *G. theta* reveal plenty of genes maintaining functions of the nucleomorph and periplastidal space [10,11]. Sequencing the nucleomorph chromosomes uncovers genes encoding plastid function [10,16,21]. One of these is a 477 bp open reading frame (ORF) with significant homology to prokaryotic rd genes. In order to examine transcription of the rd ORF, we screened a cDNA library of *G. theta* yielding a polyadenylated cDNA of 575 bp.

In comparison to prokaryotic rd sequences, the deduced amino acid sequence of the *G. theta* protein contains three striking differences. (1) An NH_2-terminal extension: we assume that the amino acids 1–56 may act as a transit peptide to direct the preprotein into the stroma of the chloroplast. In spite of the unusual amino acid composition in comparison to transit peptides of higher plants and green algae, the NH_2-terminal stretch of nucleomorph-encoded rd contains many serines and hydrophobic or aliphatic amino acids. This divergence from higher plants transit peptides has been observed for several nucleomorph-encoded proteins with plastid functions for example FtsZ and GroEL [16,21]. Remarkably, the only other known rd with an NH_2-terminal extension (showing no sequence homology to nucleomorph rd NH_2-terminus) can be found in the higher plant *Arabidopsis*, where recently an EST sequence encoding a hypothetical rd has been deposited in the data bank (AAD25628, [22]). (2) A putative membrane anchor: the very COOH-terminal stretch of nucleomorph rd, common to the recently published cyanobacterial rds from *Synechocystis* and *Anabaena* as well as *Arabidopsis* rd, is predicted as a hydrophobic 22 amino acid transmembrane helix that resembles a typical membrane anchor (predicted by SOSUI, http://www.tuat.ac.jp/–mitaku/adv_sosui/). The fact that all rd proteins from aerobic, photosynthetically active organisms carry a putative membrane anchor suggests association of the protein with the thylakoid membrane. In contrast to other iron–sulfur proteins, for example ferredoxins, no labile sulfur is present in rd-type iron centers, imposing no restriction to the localization of the redox reactive site of the nucleomorph rd. Two locations are possible for the active site of the molecule, the lumen of the thylakoids or the stroma. Functions in the stroma are more likely, however, for example in alkane hydroxylation or xanthophyll biosynthesis, which occur in the stroma associated to the thylakoid membrane [23]. In addition, it seems also possible that 'anchored' rds play a role as electron carriers in aerobic photosynthesis itself. (3) Further clues for a possible role of rds in photosynthesis come from a unique linker region only present in rds of aerobic photosynthetic organisms. In addition to the putative membrane anchor, nucleomorph rd as well as rds from cyanobacteria and higher plants contain an about 20 amino acids long region of unknown function linking the typical rd fold to the membrane anchor (Fig. 1). These linkers show very high sequence identity (more than 65% to nucleomorph rd in aver-

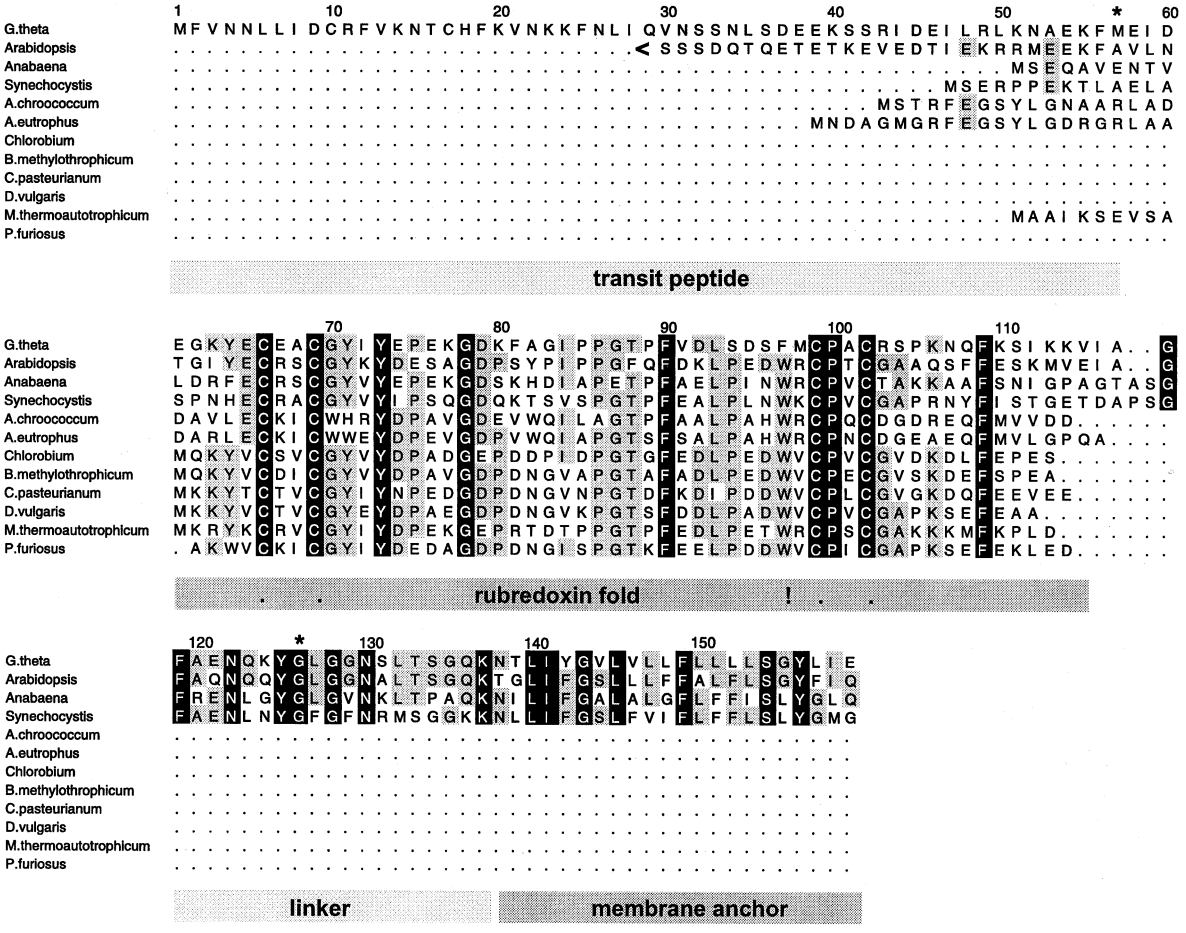

Fig. 1. ClustalW alignment [37] of rd proteins from *G. theta* (this work, CAB40406), *Arabidopsis* ([22], AAD25628), *Synechocystis* sp. strain PCC6803 ([7], BAA17090), *Anabaena variabilis* (unpublished, CAB45645), *Azotobacter chroococcum* ([38], P48343), *Alcaligenes eutrophus* ([39], P31912), *Chlorobium limicola* ([40], P09947), *Butyribacterium methylotrophicum* ([41], P14071), *C. pasteurianum* ([42], P00268), *D. vulgaris* ([43], P00269), *Methylobacterium thermoautotrophicum* ([44], AAB84661) and *P. furiosus* ([45], P24297) using the 'blosum62' weight matrix, a gap opening penalty of 20 and a gap extension penalty of 0.1. Conserved amino acids in all sequences are shaded black, conserved amino acids in at least 50% of the compared sequences are shaded gray. The cysteine ligands of the iron ion are marked with ●, ! marks residue 97, which is a phenylalanine instead of a tryptophan in all known rds so far. The boundaries of the recombinant nucleomorph rd cloned and expressed in this work are indicated with *. The first 65 amino acids of the hypothetical *Arabidopsis* rd protein are not displayed (marked with <). Possible functions of parts of the nucleomorph rd are shown below. Identities to nucleomorph rd (relevant amino acid positions according to nucleomorph rd in parentheses) are: *Arabidopsis* rd 44% (amino acids 30–137), *Synechocystis* sp. strain PCC6803 rd 44% (amino acids 58–138), *A. variabilis* rd 48% (amino acids 63–138), *A. chroococcum* rd 38% (amino acids 60–109), *A. eutrophus* rd 38% (amino acids 61–109), *C. limicola* rd 43% (amino acids 63–100), *B. methylotrophicum* rd 46% (amino acids 63–109), *C. pasteurianum* rd 48% (amino acids 63–114), *D. vulgaris* rd 46% (amino acids 63–111), *M. thermoautotrophicum* rd 50% (amino acids 63–112) and *P. furiosus* rd 45% (amino acids 63–113), respectively (data from Psi-BLAST [46]).

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                60        70        80        90        100       110       120
                |         |         |         |         |         |         |
AA              MEIDEGKYECEACGYIYEPEKGDKFAGIPPGTPFVDLSDSFMCPACRSPKNQFKSIKKVIAGFAENQKYG
PHD_sec               EEEEEEEEEE                                HHHHHHHHHHHHHHH
Rel_sec         94676514762154223789899987998998734798876478999895678999999999834679 9
SUB_sec         L.LLLL..EE..E....LLLLLLLLLLLLLLL..LLLLLL.LLLLLLLHHHHHHHHHHHHH...LLL

Consensus       ccccccccc?ccccceeccccccccccccccccccccccccccccccccchhhhhhhhhhh?hccccc
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Fig. 2. Secondary structure prediction of the recombinant nucleomorph rd (amino acids 57–126 of the preprotein) using the PHD method [47] or using a set of prediction algorithms (http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prediction.html). In the latter case, only the consensus of all secondary structure predictions is shown. AA, amino acid sequence; H, helix; E, extended (sheet); blank, other (loop); PHD, profile network prediction Heidelberg. Rel, reliability index of prediction (0 = low to 9 = high); SUB, a subset of the prediction, for all residues with an expected average accuracy >82% (L: loop, E: extended, H: helix); c, coil; e, extended (sheet); h, helix; ?, not unambiguous.

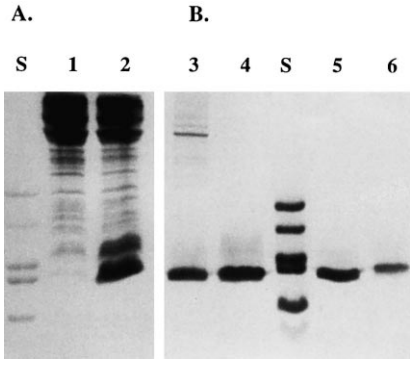

Fig. 3. 19.5% SDS–PAGE of *E. coli* BL21(DE3)/pRUB crude extracts prior to induction (lane 1) and after induction with 2 mM IPTG (lane 2). Cell lysate (soluble fraction) after DEAE chromatography (lane 3) and after gel filtration (lane 4) showing purified nucleomorph rd. Zn-rd (lane 5; retention time 21.2 min) and Fe-rd (lane 6; retention time 27.9 min) after Mono-Q chromatography. (S): fragments of horse heart myoglobin after cyanogen bromide cleavage as molecular mass marker.

age). As homology pattern searches against all data bases did not reveal other matches, we speculate that this region should have a very distinct function, perhaps as a binding motif for a particular redox partner. Together, these common features of rds of aerobic, photosynthetic organisms render these proteins members of a new family of rds.

The position of the four iron binding cysteines is identical in all rds (with exception of *Desulfovibrio desulfuricans* rd, which lacks the so-called flap region [24,25]). The consensus motif C-X-X-C-G-Y around the first two cysteines is present in nucleomorph rd, but motif C-P-X-C-G-X around cysteines 99 and 109 has a G to R substitution. Nucleomorph rd shows the strictly conserved residues Y, P and F at positions 73, 100 and 109 as well as the 86–88 PGT motif that is highly conserved among these $Fe(Cys)_4$ proteins. Residue 97 of the nucleomorph rd is F instead of W in all known rds so far. Acidic amino acids at the NH_2-terminus (E58, D60, E61, E65, E67) and two lysines at positions 113 and 114 are found in mature nucleomorph rd, while basic residues at the NH_2-terminus and acidic residues at the carboxy-terminus are frequently found in prokaryotic homologues (Fig. 1). A possible reason for this unexpected inversion may be the requirement of a recognition site for a stromal processing peptidase, necessary to cut off the transit peptide of the preprotein.

3.2. Heterologous nucleomorph rd expression

In order to overcome the very slow growth rates of *G. theta* as well as the need to isolate nucleomorph rd from the original organism, a portion of the nucleomorph rd gene corresponding to amino acids 57–126 was PCR-amplified and cloned into pET28a for overexpression in *E. coli*.

Residues 57–126 include the typical rd fold as deduced from comparison with the structurally characterized *C. pasteurianum* rd [26,27], that shows 48% sequence identity to nucleomorph rd within the rd core fold. The choice of our construct in the regions flanking the core fold is based on the following considerations: (1) residues 1–56 of the mature nucleomorph rd were removed, as they correspond to the putative transit peptide. (2) Residues 57–61 were retained to avoid disruption of possible electrostatic interactions. Data exist for *Pyrococcus furiosus* rd showing that electrostatic interactions between NH_2- and COOH-terminal residues play an important role in stabilization of both terminal β-strands [28]. In nucleomorph rd, amino acids E58, D60, E61 and K110, K113, K114 are likely candidates for this function. (3) As shown in Fig. 2, secondary structure prediction algorithms predict α-helical conformation for residues 108–121 (consensus of a set of algorithms) or for residues 106–120 of nucleomorph rd (PHD method) with very high reliability scores for amino acids 110–118. This predicted α-helix overlaps residues F109–S111, which form the COOH-terminal β-strand in homologous proteins. Further structural studies are required to decide whether nucleomorph rd shows a different folding pattern in this region. (4) For facilitated protein preparation, our recombinant nucleomorph rd lacks amino acids 127–159 including the hydrophobic membrane anchor and the second part of the linker region. The transmembrane helix was re-

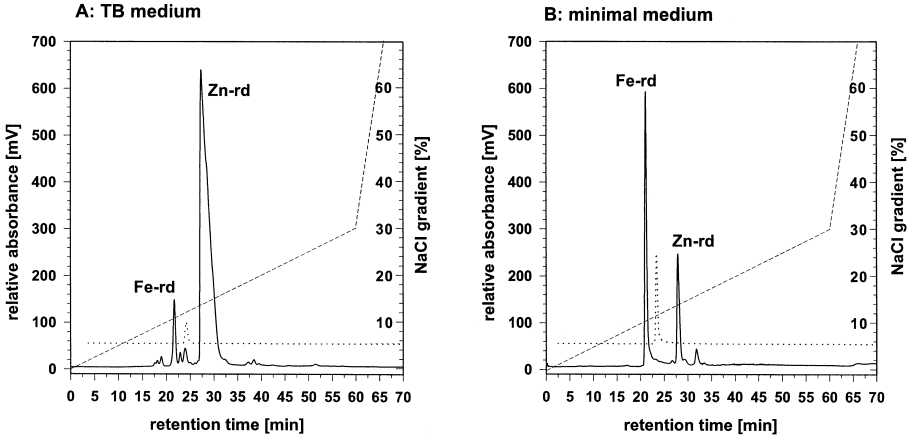

Fig. 4. Elution profiles of recombinant nucleomorph rd from different culture media. Cells were grown either in TB media (A) or in minimal media without additional zinc salts (B). Purified nucleomorph rd was subjected to anion exchange chromatography using a Mono-Q HR 10/10 column and eluted with a gradient of NaCl (dashed line; 30% corresponds to 300 mM NaCl) in 50 mM Tris–HCl (pH 8.5) at a flow rate of 1 ml/min. The solid line indicates the relative absorbance at 280 nm showing peaks for Fe-containing holorubredoxin (Fe-rd) at 27.9 min and Zn-substituted rd (Zn-rd) at 21.1 min, respectively. The dotted line indicates the relative absorbance at 490 nm showing a single peak at 21.1 min according with Fe-rd. For the sake of clarity, an offset of +50 relative absorptions units and +2.5 min retention time has been applied to the spectrum recorded at 490 nm.

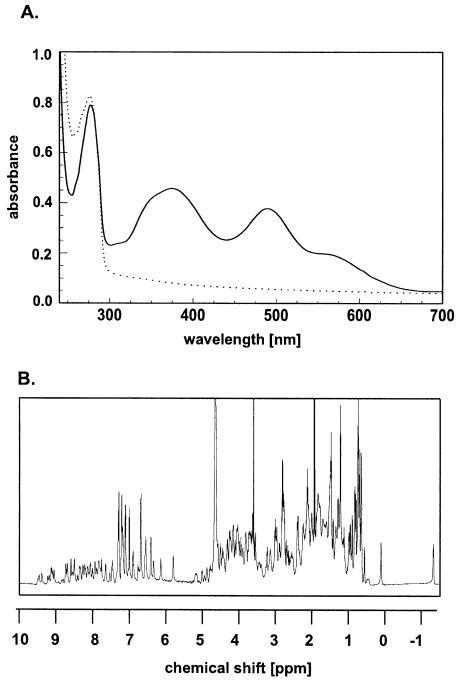

Fig. 5. (A) UV and visible absorption spectra of purified recombinant nucleomorph rd. Absorption spectra were recorded at room temperature. The solid line indicates recombinant oxidized nucleomorph holorubredoxin (Fe form) and the dotted line indicates Zn-substituted nucleomorph rd. Protein concentrations were about 50 μM for Fe-rd and 150 μM for Zn-rd. (B) 1D ^{1}H-NMR spectrum of Zn-substituted nucleomorph rd. The spectrum was recorded with a sweep width of 8389.3 Hz at 25°C, pH 6.5, in water. 10% D_2O was added as a lock signal.

moved in order to avoid (possible) unfavorable effects like low solubility and the tendency to aggregate. The second half of the conserved linker region of nucleomorph rd is absent in pRUB due to the lack of sequence information from homologous systems at the time when this construct was made.

3.3. Purification of recombinant nucleomorph rd

After induction with IPTG, good expression levels of heterologous nucleomorph rd, lacking the leader peptide as well as the putative membrane anchor, could be demonstrated by SDS–PAGE (Fig. 3A, lanes 1 and 2). Nucleomorph rd was isolated from crude cell extract by anion exchange chromatography of the supernatant on a weak anion exchanger (DEAE) followed by gel filtration and a subsequent anion chromatography step using a strong anion exchanger (Mono-Q). Rd with a purity over of 95% could thus be obtained (Fig. 3B).

Overexpression of rd genes in *E. coli* yields Fe (red) and Zn (colorless) forms of rd [29,30]. Separation of these two rd forms by Mono-Q-Sepharose chromatography is possible [31]. The ratio of Fe- to Zn-containing nucleomorph rd strongly depends on the supplied culture media (Fig. 4). As mentioned in Section 2, different ε_{280} values for the concentration determination of zinc- (5120 M^{-1} cm^{-1}) and iron- (17 190 M^{-1} cm^{-1}) containing rd have to be taken into account. Therefore, about 98% of the protein is zinc-substituted, if cell growth is done in TB media (Fig. 4A), whereas the yield of Fe-rd rises to nearly 40%, if cells are grown in minimal media containing Fe(III)-citrate but no zinc salts (Fig. 4B).

The yield of purified recombinant nucleomorph rd was about 20 mg/l for cells grown in TB media and about 15 mg/l minimal media making this protein a promising candidate for further biochemical and structural studies. The relative molecular mass of recombinant nucleomorph rd apoprotein determined by MALDI-TOF-MS was 7781, consistent with the value calculated from the amino acid sequence (7775.8 for unblocked nucleomorph rd apoprotein). The NH_2-terminal 10 residues of recombinant nucleomorph rd were identical to those predicted from the nucleotide sequence.

3.4. Spectroscopic properties

The red Fe form of nucleomorph rd showed absorption maxima in the UV and visible regions at wavelengths of 280, 390 and 490 nm (Fig. 5A). This spectrum is similar to those of native rd from *C. pasteurianum* [30] and to those of native or recombinant rds from *Desulfovibrio vulgaris* [32,33], suggesting the presence of an intact metal center. The spectrum of the colorless zinc-substituted nucleomorph rd, however, shows only one maximum at 280 nm.

The 1D NMR spectrum of Zn-rd (Fig. 5B) suggests the presence of a stable tertiary fold including β-sheet-type secondary structure as evidenced by the large dispersion of the amide proton resonances (∼7.0–9.6 ppm) and the presence of C_α proton resonances downfield from the water signal (∼4.7–5.4 ppm) [34]. The dispersion of the resonances is similar to that reported previously for a zinc-substituted form of *P. furiosus* rd [28].

The significant upfield shift of the resonances of two methyl groups (∼0.2 ppm and −1.2 ppm) most probably arises from ring current effects of the aromatic cluster in the hydrophobic rd core. Similar upfield shifts have also been reported for the conserved I33 of *C. pasteurianum* rd [35,36]. These findings support the notion that the overall fold of nucleomorph rd is similar to that of published rd structures.

Further studies, particularly the determination of the three-dimensional structure of recombinant nucleomorph rd in solution, are initiated. Surface and charge distribution analysis of the nucleomorph rd structure will give further information about cellular functions, localization and even the degree of homology to prokaryotic rds, and the localization of native nucleomorph rd in *G. theta* using anti-nucleomorph rd antibodies will yield insights into the function of this multifunctional protein in eukaryotic organisms.

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