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Synthesis and characterization of a 25-residue rubredoxin(II)-like metalloprotein and its valine-leucine mutant

Hans E.M. Christensen[a,*], Jan M. Hammerstad-Pedersen[a], Arne Holm[b], Peter Roepstorff[c], Jens Ulstrup[a], Ole Vorm[c] and Søren Østergård[b]

[a]*Chemistry Department A, Building 207, The Technical University of Denmark, 2800 Lyngby, Denmark,* [b]*Department of Chemistry, Centre for Medical Biotechnology, The Royal Veterinary and Agricultural University, 1871 Copenhagen, Denmark* and [c]*Department of Molecular Biology, Odense University, 5230 Odense, Denmark*

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An iron-sulfur metalloprotein containing the 5–12 and 35–50 residues of *Desulfovibrio gigas* rubredoxin has been synthesized by Fmoc solid phase peptide synthesis and subsequent peptide folding. A Gly links the two residue chains between Val-5 and Glu-50. Sybyl Tripos structure optimization indicates only minor structural changes of the folded synthetic protein compared to the similar residue positions in the native protein. The UV-VIS spectrum of the reduced synthetic protein is very similar to that of native *D. gigas* rubredoxin and the molecular mass determined by laser mass spectrometry has the expected value (± 2D). No metal is transferred to the gas phase by the laser beam merely by mixing the peptide and iron(II), substantiating that the folding procedure is a necessary pre-requisite for protein formation. The Val → Leu[41] chemical mutant has also been synthesized and behaves in a closely similar fashion.

Metalloprotein; Rubredoxin; Synthesis; Characterization; Chemical mutant

Electron transfer patterns of redox metalloproteins such as cytochromes, iron-sulphur proteins and blue copper proteins are frequently characterized by their long-range nature and specific surface sites for electron exchange [1,2]. Details of the electron transport routes are documented by a variety of techniques among which chemical modification at specific amino acid residues [1] and residue exchange using site directed mutagenesis [3–8] have become powerful tools.

We provide here results based on an alternative approach directed towards chemical metalloprotein synthesis, or synthesis of large parts of the proteins and their mutants. A chemical approach which incorporates solid-state peptide synthesis and suitable folding procedures offers similar perspectives as genetically engineered proteins in relation to molecular details of protein structure–function relations. This would relate for example to details of long-range, directional electron transport of redox metalloproteins, specific dependence on the intermediate protein matter, structural surface sites etc. In addition, in chemical metalloprotein synthesis not only the sequence but also the size of the protein can be controlled. This offers interesting perspectives complementary to the microbiological approach, in relation to design of artificial proteins with specific, predetermined properties.

There are several reports of peptide complexes where short peptide segments have been attached to $[Fe(Cys)_4]$ rubredoxin-like and to $[Fe_2S_2]$, $[Fe_3S_x]$ and $[Fe_4S_4]$ ferredoxin-like cores [9–15]. Semi-synthesis of cytochrome *c* has also been reported [16]. Recently the first total synthetic 55-residue $2[Fe_4S_4]$ ferredoxin (*C. pasteurianum*) [17], the 45-residue racemic rubredoxin (*D. desulfuricans*) [18], and the 104-residue horse heart cytochrome *c* [19] have been reported, with several physical properties closely matching those of the native proteins. In the present work we report the synthesis of a *D. gigas* rubredoxin analogue and its Val → Leu[41] variant (synthetic 'mutant'), both with 25 residues. We have aimed towards large enough proteins that details of protein structure–function relations can be expected to carry over to the real protein. On the other hand, in order to achieve facile peptide synthesis a loop and a terminal random coil element of expectable less primary importance in the 52-residue protein have been omitted.

Our metalloprotein design is shown in Fig. 1. Fig. 1a shows a ribbon view of native *D. gigas* rubredoxin selected as a well characterized, small metalloprotein [20,21]. The most important structural elements are the 5–12 and 35–50 fragments consisting of altogether 24 residues. The remaining sequence is composed of a 13–34 connecting loop, and the 1–4 and 51–52 terminals. The shortest gap left when these segments are cut is 0.54 nm between Val[5] and Glu[50]. This gap can be closed by

Correspondence address: A. Holm, Department of Chemistry, Centre for Medical Biotechnology, The Royal Veterinary and Agricultural University, 1871 Copenhagen, Denmark.

Present address: Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, 0511 Singapore.

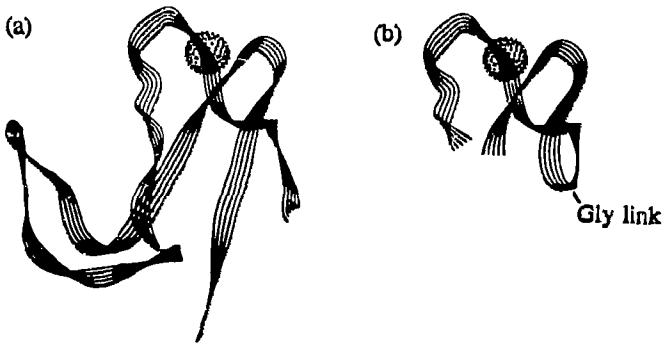

Fig. 1. Ribbon representation of *D. gigas* rubredoxin (left) and of the optimized structure of the synthetic 25-residue iron(II)-sulfur peptide. Rubredoxin coordinates from ref. 19 and Brookhaven Data Bank [21]. Evans and Sutherland graphics.

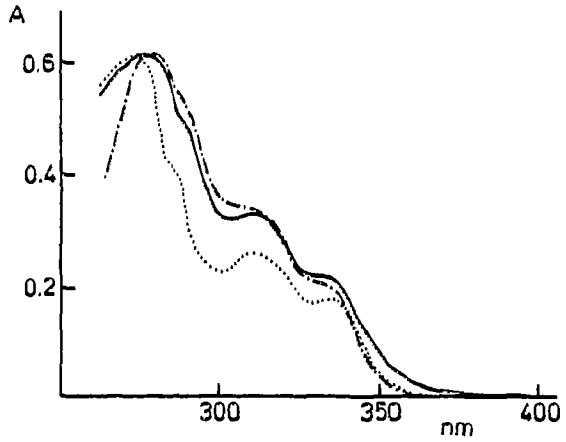

Fig. 2. Absorption spectra of the 25-residue *D. gigas* rubredoxin iron(II)-sulfur analogue (———), its Val → Lys[41] variant (–·–·) and of native *C. pasteurianum* rubredoxin(II) [24] (·····). The spectra are normalized with respect to the 278 nm Tyr absorption maximum.

a Gly residue (0.38 nm). After energy minimization using Sybyl Tripos software the structure in Fig. 1b is obtained. The charge of this modified protein would be −6 at pH 7 compared to −8 for the native protein.

The coordinating units in both *G. gigas* and *D. vulgaris* rubredoxins are Cys[6]-Thr[7]-Val[8]-Cys[9] and Cys[39]-Pro[40]-Val[41]-Cys[43] [15,20,22]. The former is conserved in *C. pasteurianum* while the latter sequence here is Cys[39]-Pro[40]-Leu[41]-Cys[42]. In view of this an appropriate first choice of synthetic variant is Val → Leu[41].

The peptide chains consisting of the *D. gigas* rubredoxin 1–12 and 35–50 residues linked at Val[5]-Glu[50] by Gly (H-DDWACPVCGASKDAFEGVCTVCGYE-OH), and its Val → Leu[41] variant (-PVC- → -PLC-) were synthesized by solid phase peptide synthesis. Fmoc α-amino protecting strategy was used [23]. The peptides were purified by reverse phase HPLC. Mass spectrometric and amino acid analysis were in agreement with the assigned structures.

The purified synthetic protein (0.5 mg) was dissolved in 8 M urea (0.5 ml, pH 8.5, 4 mM Tris, 1 mM dithiothreitol (DTT)) and 20 μl iron(II) sulphate solution added to give 2–10 times excess of iron. The protein

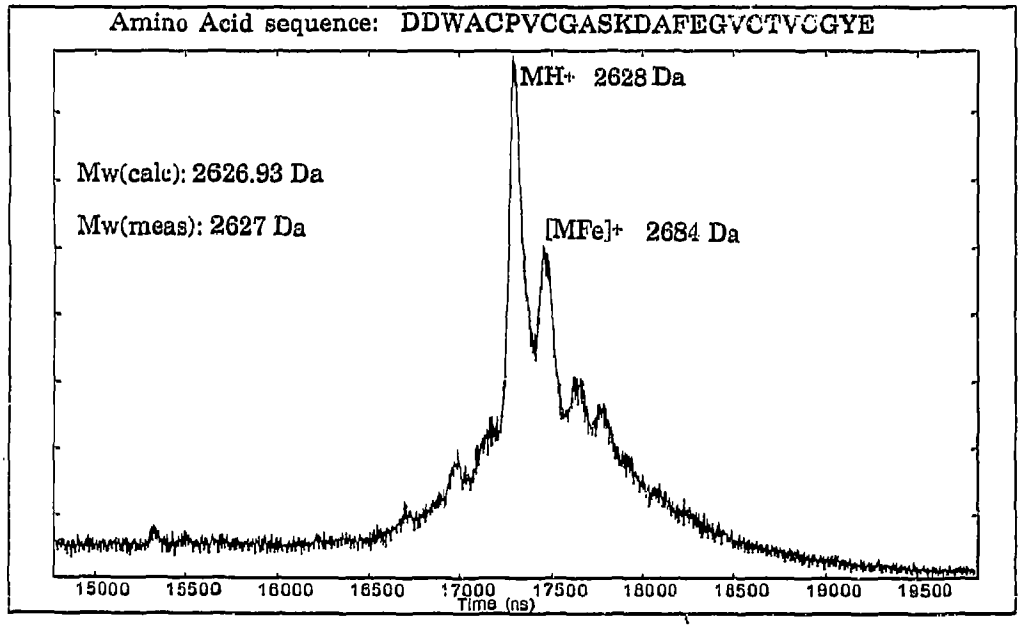

Fig. 3. Matrix assisted laser desorption mass spectra of the free peptide peak (peak of lower molecular weight) and the peptide/iron complex (peak of higher molecular weight). The MW accuracy is ± 2D.

folded around Fe^{2+} as the urea concentration was slowly lowered to less than 1 M by titrating under gentle stirring a 4 mM Tris (pH 8.5, 1 mM DTT) solution into the Fe(II)/peptide solution (1 ml/H) in an argon atmosphere. The process could be followed by recording the absorption spectrum of the solution as folding progressed, using a Milton Roy diode-array spectrophotometer.

After completion of the folding, the reaction mixture showed two FPLC peaks (Pharmacia Biosystem). The first, larger peak absorbs at both 278 and 335 nm and is assigned to the Fe(II)/peptide complex. The second one absorbs only at 278 nm. Blank experiments on Fe(II)-free peptide solutions gave only the latter peak which is therefore assigned to unfolded free peptide.

The synthetic proteins in aqueous solution were characterized by their UV-VIS spectra and their molecular weight determined by matrix assisted laser mass spectrometry (MALD-MS) [25]. UV-VIS spectra of the 'native' and mutant-folded metalloproteins in their reduced forms are shown in Fig. 2. The 278 and 290 bands of Tyr and Trp and FeS_4 bands at 311 and 333 nm appear clearly. Both band maxima and relative absorbances correspond well to those of the native rubredoxin, indicative of basically correct folding and coordination.

Analysis of the native rubredoxin analogue by MALDI-MS, using 2,5-dihydroxy benzoic acid as matrix [26] and low laser fluence for desorption/ionization shows two prominent peaks (Fig. 3), one stemming from the peptide and one corresponding to a peptide/iron complex. The fact that stable peptide/iron ions can be formed strongly indicates that the peptide has actually folded around iron, since non-covalent, even high-affinity protein–metal association is most frequently observed to be labile in MALDI-MS [27]. Several other observations strongly indicate that the observed peptide/iron are unlikely to be caused by unspecific adduct ions arising from solid-state or gas phase association. The peptide/iron peaks thus disappear with increasing fluency, they are less prominent in spectra from sinapinic acid matrices [28], and most importantly, peptide-iron ions could not be obtained from the unfolded peptide in the presence of even large excess of Fe^{2+}.

In conclusion the absorption spectra, the FPLC behaviour, and the mass spectrometric analysis all indicate that a 25-residue Fe-S peptide of correct folding, coordination geometry and molecular weight is formed when folding can be controlled. The UV/VIS spectrum of the Val → Lys^{41} variant furthermore indicates that synthetic mutant proteins can be formed under similar conditions. The data are finally supported by structure optimization which indicates that only small and 'evenly' distributed structural changes have occurred relative to the structure of native *D. gigas* rubredoxin.

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[26] All LD mass spectra were recorded using a BIO-ION BIN 10K PDMS instrument modified for use in MALDI MS. The samples were prepared by mixing 1 μl of peptide solution (0.1 μg/μl in a buffer containing 4 mM Tris, 1 mM DTT, 1 M urea, and 0.13 μg/μl of Fe(II) sulfate heptahydrate) with 9 μl of either matrix

solution (2,5-dihydroxy benzoic acid: 10 μg/μl. Sinapinic acid: 10 μg/μl in 30% acetonitrile/70% water). A 1 μl aliquot of the mixture was transferred to a stainless-steel target and allowed to dry at room temperature.

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[28] Desorption/ionization from the 2,5-dihydroxybenzoic acid matrix produces 'colder' ions, i.e. ions of less internal energy than for example sinapinic acid and ferulic acid matrices (B. Spengler et al., poster presented at the 12th Int. Mass Spectrometry Conf., 1991, Amsterdam). The peptide-metal bond is therefore more likely to remain intact in the former than in the latter matrices.