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Ambineela, an Unusual Blue Protein Isolated from the Archaeon *Acidianus ambivalens*

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A novel blue protein, named ambineela, was isolated from the soluble extract of the thermoacidophilic archaeon *Acidianus ambivalens*. In solution, the purified protein is a monomer with 50 kDa and has a basic character (pI ~8.7). The electronic spectrum shows two bands, centred at 395 and 625 nm (A_{625}/A_{395} = 0.7). The protein does not contain any transition metal; its blue colour is due to an unidentified non-fluorescent cofactor, covalently bound to it. Ambineela N-terminal sequence exhibits a consensus ADP-binding region, suggesting that its unknown cofactor may comprise this molecule or an analogue.

Key Words: **Archaea; thermophile; ADP; flavin.**

The archaeal domain is extremely diverse in respect to metabolic strategies, protein chemistry and structure. The analysis of the complete genome sequences of the archaea *Methanococcus janaschii* [1], *Archaeoglobus fulgidus* [2] and *Methanobacterium thermoautotrophicum* [3], has provided an extended illustration of this situation. For example, a quarter of *A. fulgidus* genome encodes for new proteins while another quarter encodes for functionally uncharacterised but conserved proteins [2]. In terms of protein cofactors, archaea can also be rather diverse from eubacteria. A good example is given by the methanogens, whose proteins contain several strictly archaeal coenzymes [4]. Also, tungsten, an element not so widely found among eubacteria, appears to be an element that may play a key role in the metabolism of heterotrophic hyperthermophiles, as *Pyrococcus furiosus* [5]. Unique respiratory quinones are also found among archaea, in particular in the sulfur metabolising *Sulfolobales*, which are derivatives of benzo-[b]-thiophen-4,7-quinone, all bearing a saturated C_{30} isoprenoid side chain [6]. Surely many other protein cofactors and metabolites remain to be found and characterised.

In the course of our investigations on metalloproteins from archaea, we have been studying proteins isolated from the thermoacidophile *Acidianus ambivalens* [7-11]. This organism is a chemolitoautotroph, that grows optimally at pH 2.5 and 80°C. Under aerobic conditions, it oxidises elemental sulfur with oxygen, producing sulfuric acid; anaerobically, it reduces elemental sulfur with hydrogen, forming hydrogen sulfide [12,13]. On the present work we report the isolation and partial characterisation of a soluble blue protein found in this organism, here named Ambineela (Ambi, from the organism name *A. ambivalens* and neela, from the Sanskrit word for blue).

MATERIALS AND METHODS

Cell growth and protein purification. *A. ambivalens* (DSM 3772) cell mass and soluble extract were obtained as previously described [8]. The soluble extract, after dialysis against water, was loaded into a DEAE-cellulose column and eluted as described [8], with the exception that the working pH was 6.5, using 10 mM piperazin as buffer. The subsequent steps were performed on a Pharmacia HiLoad system. During the purification, ambineela was tracked by following its broad absorption band at 625 nm. The protein eluted from the DEAE column at ~20 mM NaCl, being pooled, concentrated and applied into a Superdex S-200 column (1.6 L). This column was run at 1.6 $ml.min^{-1}$ and ambineela was eluted with 10 mM piperazin pH 6.5, 150 mM NaCl. The fractions pooled from the gel filtration step, were dialysed and injected into an SP Sepharose column (12 × 1.6 cm), which had been equilibrated at 1 $ml.min^{-1}$ with 10 mM MES, pH 6.0 . A linear gradient from 0 to 500 mM NaCl was applied at 1 $ml.min^{-1}$ and ambineela eluted at ~50 mM NaCl. The protein was pooled, concentrated and loaded into a gel filtration Superdex 75 column (60 x 2.6 cm). This column was run at 1 $ml.min^{-1}$ and the buffer was the same as the one used in the first gel filtration. After this step, the protein (< 1mg) was pure, as judged by the presence of a single band in a Comassie Blue stained 12.5% SDS/Page, and by the clean N-terminal sequence obtained.

Biochemical and spectroscopic procedures. X1 Protein concentration was determined by the Bradford method [14]. The protein molecular mass was determined by 12.5 % SDS/PAGE and by gel filtration using a Superose 12 (Pharmacia) column and the Boehringer Combithek kit proteins as standards. Ultraviolet-visible spectra were recorded on a Beckman DU-70 spectrophotometer, equipped with a temperature controller. EPR spectra were recorded as in [8]. The protein N-terminal sequence was determined using an Applied Biosystem Model 470A sequenator. Search of protein sequences showing homology with the N-terminus was performed at the NCBI using the BLAST network service. Secondary structure predictions were made with the program PREDATOR [15]. Metal content was deter-

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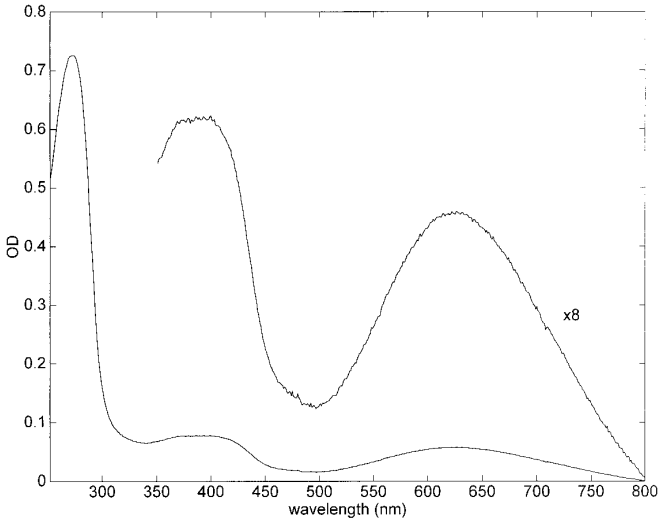

FIG. 1. *A. ambivalens* ambineela UV-visible spectra.

mined by plasma emission at ITQB and by atomic absorption in a graphite chamber at the Laboratório de Análises, IST, Lisbon. Protein concentration was determined by the Bradford method [14]. The protein molecular mass was determined by 12.5 % SDS/PAGE and by gel filtration using a Superose 12 (Pharmacia) column and the Boehringer Combithek kit proteins as standards. Ultraviolet-visible spectra were recorded on a Beckman DU-70 spectrophotometer, equipped with a temperature controller. EPR spectra were recorded as in [8]. The protein N-terminal sequence was determined using an Applied Biosystem Model 470A sequenator. Search of protein sequences showing homology with the N-terminus was performed at the NCBI using the BLAST network service. Secondary structure predictions were made with the program PREDATOR [15]. Metal content was determined by plasma emission at ITQB and by atomic absorption in a graphite chamber at the Laboratório de Análises, IST, Lisbon.

RESULTS AND DISCUSSION

A blue protein was completely purified from the soluble extract of *A. ambivalens*. The protein is a monomer with a molecular mass of 50 kDa, as determined from gel filtration and SDS/Page. Isoelectric focusing revealed that the protein is basic, having a pI of 8.7. Metal analysis revealed that no biologically relevant transition metals (V, Mn, Fe, Co, Ni, Cu, Mo, W) were present in pure ambineela, a surprising feature taking into account its colour. The visible spectrum of ambineela is shown in Fig. 1. Two main features are observed, namely bands at 625 nm and 395 nm, in a ratio of ~ 0.7. Chemical reduction of the protein with sodium dithionite leads to a bleaching of the visible spectrum, with no additional bands observed. This is a reversible process, since reoxidation of the sample does restore its initial spectral characteristics. Thus, ambineela may be involved in an as yet unknown redox process. Anaerobic incubation with NADH (35 μM), NADPH (35 μM), sodium sulfide (80 μM) and sodium ascorbate (10 mM) had no effect on the protein spectral features. The protein is stable in a wide pH range, from pH 4.0 to pH 9.0, as evaluated from its unaltered visible spectrum. The protein is not fluorescent, as judged by the absence of emission upon excitation at 395 or 625 nm, even in the presence of 80% dimethyl sulfoxide (DMSO) or when using dioxane as solvent. Ambineela was found to be EPR silent, both in the oxidised and dithionite reduced forms.

In order to try to identify the nature of ambineela cofactor, its extraction was attempted using several conditions and solvents. After protein precipitation with 20% tricloroacetic acid and centrifugation, the resulting supernatant exhibits no visible spectral features. Chemical extractions using ethyl acetate, carbon tetrachloride and dichloromethane were also attempted, but again, visible spectroscopy did not reveal the presence of any cofactor in the organic phase. Also, the cofactor was neither released nor the spectrum altered by 80% DMSO.

The N-terminal sequence of *A. ambivalens* ambineela was determined up to residue 49 (Fig. 2). Screening of databases for analogous proteins revealed homologies towards several different flavin containing proteins from *Eubacteria*, like *Rhodobacter capsulatus* sulfide quinone reductase and *Escherichia coli* NADH dehydrogenase, among several others. Regarding archaeal proteins, a putative protein from *Archaeoglobus fulgidus*, similar to *R. capsulatus* sulfide-quinone oxidoreductase, also contains an N-terminus similar to that of ambineela. All these proteins have $\sim$50% similarity towards ambineela N-terminus. The common feature between these proteins lies in the fact that they all contain a consensus ADP-binding $\beta\alpha\beta$-unit in their N-terminal region [16] (Fig. 2, grey boxes). This motif spans over approximately 30 aminoacid residues that are predicted to fold in a $\beta\alpha\beta$ manner, and interestingly, they occur near the N-terminus [16]. This is also the case in ambineela, in which only one residue deviates from the fingerprint (residue 34) [16]. Moreover, prediction of the secondary structure points to a $\beta\alpha\beta$ arrangement (Fig. 2). Taking into account the results of the N-terminus analysis, the hypothesis that ambineela could contain a flavin that dissociated during protein purification was put. However, incubation of pure ambineela with either FAD or FMN in the presence of dithiotreitol, followed by dialysis, did not result in flavin insertion, as evaluated by visible spectroscopy.

Blue coloured, non-metal containing proteins are not very usual in biological systems. Other proteins that fit into this category are some algal and cyanobacterial biliproteins that contain phycocyanobilin, a blue-coloured pigment. However, its spectral properties are dissimilar to those found in ambineela: phycocyanobilins have sharp, intense bands in the 600-670 nm region and fluorescence emission at $\sim$680 nm [17]. Antocyanins which can also exhibit a blue colour, were other candidate pigments. They can be identified in a simple

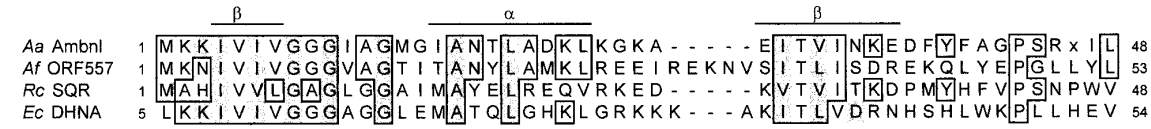

FIG. 2. Ambineela N-terminus sequence comparison. *Aa* Ambnl, *A. ambivalens ambineela; Af* ORF557, *Archaeoglobus fulgidus* ORF 557; *Rc* SQR, *Rhodobacter capsulatus* sulfide-quinone oxidoreductase and *Ec* DHNA, *Escherichia coli* NADH dehydrogenase.

fashion by acidification to pH 1.0, condition in which the chromophore blue band is red-shifted. However, the presence of this cofactor in ambineela was ruled out, since in these conditions, no new spectral features were observed in the electronic spectrum of ambineela. The blue-type flavin semiquinones also exhibit broad bands in the 600 nm region, but these are transient species, stabilised by protein during reduction [18]. If this was the case in ambineela, the observed spectral features would change in the presence of oxidants, which was not the case, since ammonium persulfate had no effect on the visible spectrum. Moreover, a radical species at g 2.00 would be observed in the EPR spectrum. Chemically synthesised mercaptoflavins, once incorporated into apo-flavoproteins—a strategy used to probe flavin binding domains [19], also exhibit a broad band in the blue region but may be fluorescent. To our knowledge, no naturally occurring mercaptoflavoprotein has ever been isolated.

The present set of data does not allow us to establish the nature of *A. ambivalens* blue protein cofactor nor the protein function. From the analysis of the N-terminal region, which elicits the presence of an ADP-binding motif, it may be inferred that the protein cofactor(s) either comprises an ADP molecule or an analogue of it capable of interacting in a similar fashion with the predicted protein fold. The possibility that ambineela contains a covalently bound mercaptoflavin cannot be excluded at this stage and would be the second example of chromophores containing thio-groups, after the benzothiophenquinones [6].

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REFERENCES

1. Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, *et al.* (1996) *Science* **273,** 1058–1073.
2. Klenk, H.-P., Clayton, R. A., Tomb, J.-F., White, O., Nelson, K. E., *et al.* (1997) *Nature* **390,** 364–370.
3. Smith, D. R., Doucette-Stamm, L. A., Deloughery, G. Lee, H., *et al.* (1997) *J. Bacteriol.* **179,** 7135–7155.
4. Daniels, L. (1993) *In* The Biochemistry of Archaea (M. Kates, , D. J. Kushner, and A. T. Matheson, Eds.), Elsevier.
5. Adams, M. W. W. (1993) *Annu. Rev. Microbiol.* **47,** 627–658.
6. Tricone, A., Lanzotti, V., Nicolaus, B., Zillig, W., De Rosa, M., and Gambacorta, A. (1989) *J. Gen.Microbiol.* **135,** 2751–2757.
7. Anemüller, S., Schmidt C. L., Pacheco, I., Schäfer, G., and Teixeira, M. (1994) *FEMS Microbiol. Lett.* **117,** 275–280.
8. Teixeira, M., Batista, R., Campos, A. P., Gomes, C. M., Mendes, J., Pacheco, I., Anemüller, S. and Hagen, W. (1995) *Eur. J. Biochem.* **227,** 322–327.
9. Bentrop, D., Bertini, I., Luchinat, C. Mendes, J., Piccioli, M., and Teixeira, M. (1995) *Eur. J. Biochem.* **236,** 92–99.
10. Giuffrè, A., Gomes, C. M., Antoninni, G., D'Itri, E., Teixeira, M., and Brunori, M. (1997) *Eur. J. Biochem.* **250,** 383–388.
11. Gomes, C. M., and Teixeira, M. (1998) *Biochim. Biophys. Res. Commun.* **243,** 412–415.
12. Zillig, W., Yeats, S., Holz, I., Böck, A., Rettenberger, M., Gropp, F., and Simno, G. (1986) *Syst. Appl. Microbiol.* **8,** 197–203.
13. Fuchs, T., Huber, H., Burggraf, S., and Stetter,K. O. (1996) *Syst. Appl. Microbiol.* **19,** 56–60.
14. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
15. Frishman, D., and Argos, P. (1995) *In* Proteins: Structure, Function and Genetics, Vol. 23, pp. 566–579.
16. Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) *J. Mol. Biol.* **187,** 101–107.
17. Sidler, W. A. (1994) *In* Advances in Photosynthesis, The Molecular Biology of Cyanobacteria (D. A. Bryan, Ed.), Kluwer Academic, Dordrecht.
18. Massey, V., and Hemmerich, P. (1980) *Biochem. Soc. Trans.* **8,** 246–257.
19. Moore, E., Ghisla, S., and Massey, V. (1979) *J. Biol. Chem.* **254,** 8173–8178.