View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

FEBS Letters 580 (2006) 6151–6154

Nitrogenase Fe protein-like Fe–S cluster is conserved in L-protein (BchL) of dark-operative protochlorophyllide reductase from *Rhodobacter capsulatus*

Jiro Nomata[a], Masaharu Kitashima[b], Kazuhito Inoue[b], Yuichi Fujita[a,*]

[a] *Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*
[b] *Department of Biological Sciences, Kanagawa University, Hiratsuka, Kanagawa 259-1293, Japan*

Received 22 September 2006; revised 7 October 2006; accepted 9 October 2006

Available online 17 October 2006

Edited by Richard Cogdell

Abstract Dark-operative protochlorophyllide reductase (DPOR) in bacteriochlorophyll biosynthesis is a nitrogenase-like enzyme consisting of L-protein (BchL-dimer) as a reductase component and NB-protein (BchN–BchB-heterotetramer) as a catalytic component. Metallocenters of DPOR have not been identified. Here we report that L-protein has an oxygen-sensitive [4Fe–4S] cluster similar to nitrogenase Fe protein. Purified L-protein from *Rhodobacter capsulatus* showed absorption spectra and an electron paramagnetic resonance signal indicative of a [4Fe–4S] cluster. The activity quickly disappeared upon exposure to air with a half-life of 20 s. These results suggest that the electron transfer mechanism is conserved in nitrogenase Fe protein and DPOR L-protein.
© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Protochlorophyllide reductase; Nitrogenase; Bacteriochlorophyll biosynthesis; BchL; Fe–S cluster; *Rhodobacter capsulatus*

1. Introduction

Nitrogenase-like enzymes play crucial roles in chlorophyll (Chl) and bacteriochlorophyll (BChl) biosynthesis [1–3]. One nitrogenase-like enzyme is dark-operative protochlorophyllide (Pchlide) oxidoreductase (DPOR), which catalyzes the reduction of Pchlide D-ring to form chlorophyllide *a* (Chlide) [1]. By this reduction, the porphyrin ring of Pchlide is converted to chlorin ring. Spectroscopic properties of Chlide are identical to those of Chl *a*. Thus, the Pchlide reduction could be considered the final step in generating the spectroscopic properties of Chl *a*. Another nitrogenase-like enzyme, Chlide oxidoreductase (COR), participates in BChl biosynthesis [2]. COR catalyzes the reduction of Chlide B-ring, the diagonal ring of D-ring, to form 3-vinyl bacteriochlorophyllide *a*, which has a bacteriochlorin structure. Thus, the sequential actions of two nitrogenase-like enzymes, DPOR and COR, convert porphyrin (Pchlide) to bacteriochlorin (3-vinyl bacteriochlorophyllide *a*) in BChl *a* biosynthesis [2].

Nitrogenase is a complex metalloenzyme converting dinitrogen to ammonia coupling with ATP hydrolysis (for review see [4]). Fe protein and MoFe protein are crucial components of nitrogenase. Fe protein functions as an ATP-dependent reductase for MoFe protein, and MoFe protein provides a catalytic center for dinitrogen reduction. Fe protein is reduced by ferredoxin (or dithionite *in vitro*) and transfers electrons to MoFe protein, which is coupled with ATP hydrolysis. Electrons are finally transferred to dinitrogen at the catalytic site of MoFe protein resulting in the formation of ammonia molecules. Fe protein is a homodimer of NifH, and has a [4Fe–4S] cluster that bridges the NifH protomers as the metallocenter of electron transfer. Two Cys residues from each protomer (Cys98 and Cys132 in *Azotobacter vinelandii* NifH) coordinate the [4Fe–4S] cluster. Fe protein is extremely sensitive to oxygen with half-life of less than 1 min because the [4Fe–4S] cluster is irreversibly destructed by oxygen.

Some nitrogenase-like features of DPOR presumed from the sequence similarity have been experimentally shown in previous works [3,5,6]. DPOR consists of two crucial components, an ATP-dependent reductase (L-protein, BchL-dimer) and a catalytic component (NB-protein, BchN–BchB-heterotetramer), which are counterparts of Fe protein and MoFe protein, respectively. The amino acid sequence of BchL shows about 30% identity to that of NifH. The two Cys residues for the cluster chelation and ATP-binding motif are conserved in BchL [1]. It is felt from the structural features that the electron transfer mechanism of DPOR is similar to that of nitrogenase [1,5,6]. However, no metallocenters of DPOR components have been identified so far. Here we describe for the first time that L-protein from the purple bacterium *Rhodobacter capsulatus* has an oxygen-sensitive [4Fe–4S]-type cluster that resembles the Fe–S cluster of nitrogenase Fe protein. These features of Fe–S centers suggest that the ATP-dependent electron transfer mechanism to the catalytic component is conserved in diverged enzymes, nitrogenase and DPOR.

2. Materials and methods

2.1. Strains and culture conditions

A photosynthetically competent mutant DB176 of *R. capsulatus* was used as the host strain to overexpress 6 × HN- and Strep-tagged BchL proteins. DB176 transconjugants harboring BchL-overexpression vectors were grown as described [2,6].

*Corresponding author. Fax: +81 52 789 4107.
E-mail address: fujita@agr.nagoya-u.ac.jp (Y. Fujita).

Abbreviations: Pchlide, protochlorophyllide; EPR, electron paramagnetic resonance; DPOR, dark-operative protochlorophyllide oxidoreductase; Chl, chlorophyll; BChl, bacteriochlorophyll; Chlide, chlorophyllide *a*

0014-5793/$32.00 © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2006.10.014

2.2. Construction of overexpression plasmids

Two plasmids, pJN3L and pJN4L, were constructed to overexpress BchL as fusion proteins with 6 × HN-tag and Strep-tag, respectively, under the control of the *puc* promoter in *R. capsulatus* DB176 (Fig. 1(A)). The plasmid pJN3L was constructed as follows. A chimeric DNA fragment between *puc* and a tag-coding sequence was amplified by PCR pPucf [5] and pPucNHr1 (5′- TTGGTACCCCAGAGTTATGATTATGATTATGATTATGATTATGATTATGTCTCATTGTCCCGAATCCTCCAA-3′) using pJRD-SFX [6] as the template. The amplified DNA fragment was ligated into BamHI and KpnI (underlined above) sites of pBBR1MCS2 [7], yielding pJN1. A spectinomycin resistance cartridge derived from pHP45Ω [8] was ligated into the SacI and XbaI sites of pJN1, yielding pJN3. The *bchL* coding region was amplified from the *R. capsulatus* genomic DNA with the primers bchLf2 (5′-GTGGTACCAAGCCCGCGCGACGATATTCC-3′) and bchLr2 (5′-ACGGTACCTCAGTCGAATCCCAGAAGTTC-3′), and ligated into the KpnI site of pJN3, yielding pJN3L. Plasmid pJN4L was constructed as follows. Primers, bchLf3 (5′-AGTTCGAGAAGTCGGGGGTGAGCCCGCGCGACGATATTCC-3′) and bchLr2, were used to amplify the *bchL* coding region with an extension for the second PCR cycle. Another 2 kb chimeric DNA fragment containing the spectinomycin cartridge and the *puc* promoter was amplified [2]. The second PCR reaction was followed by amplification with the primers "pBBRT7f1" [2] and "bchLr2". The amplified 3 kb fragment was ligated into the KpnI site of pBBR1MCS2, yielding pJN4L. The plasmids pJN3L and pJN4L were introduced into *R. capsulatus* DB176 cells by triparental mating [6].

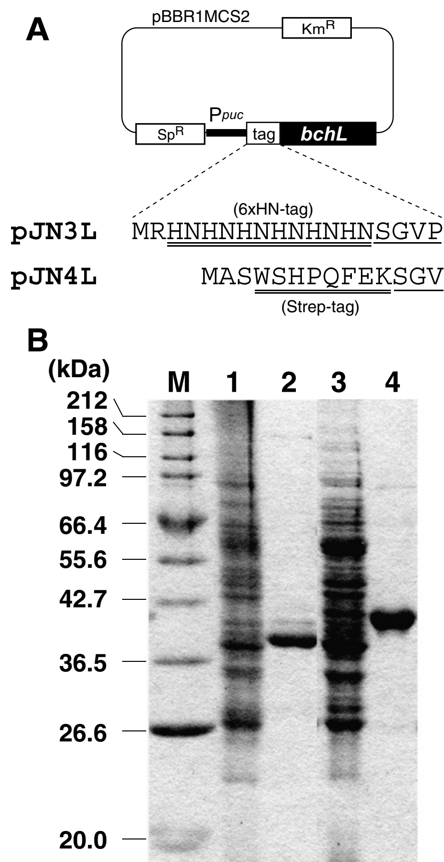

Fig. 1. (A) Plasmids for overexpression of 6 × HN- and Strep-BchLs in *R. capsulatus*. The plasmids pJN3L and pJN4L were constructed to overexpress BchL as fusion proteins with 6 × HN- and Strep-tags, respectively. (B) SDS–PAGE of purified Strep-tag and 6 × HN-tag BchLs. BchLs with 6 × HN-tag (lane 4) and Strep-tag (lane 2) were purified from the crude extracts of DB176 with pJN3L (lane 3) and pJN4L (lane 1) by Ni-NTA and Strep-Tactin affinity columns, respectively.

2.3. Purification of L-proteins

Crude extracts were prepared as described previously [6]. All procedures for L-protein purification were carried out in an anaerobic chamber (model A, Coy Laboratory Products, Grass Lake, MI). The oxygen level was kept less than 1 ppm in the chamber. Sodium dithionite (final 1.7 mM) was added just before use to remove residual oxygen [2,5,6]. BchL with 6 × HN-tag was purified as follows. About 15 ml of the crude extract was loaded onto a Ni-NTA Sepharose column (1 ml of Ni-NTA Sepharose in a 0.9 cm × 2.2 cm column, QIAGEN) that was equilibrated with the wash buffer (20 mM sodium phosphate (pH 8.0), 20 mM imidazole, 500 mM NaCl, 10 mM dithiothreitol). After the column was washed with 5 ml of wash buffer, 6 × HN-tag BchL was eluted with the elution buffer (20 mM sodium phosphate (pH 8.0), 500 mM imidazole, 500 mM NaCl, 10 mM dithiothreitol). Strep-tag BchL was purified as described previously [2]. Protein concentrations were determined using Protein Assay (Bio-Rad) with bovine serum albumin as a standard.

2.4. Assay of L-protein activity

L-protein activity of crude extracts and purified L-protein was determined by mixing with complementary crude extracts containing overexpressed NB-proteins with the respective tags in DPOR assay mixtures (20 μM Pchlide) as described [6]. Assays were carried out at 34 °C for 7 min, and the amount of formed Chlide was estimated from the absorption spectra of hexane-extracted acetone phase [6].

2.5. Absorption spectra, EPR measurements, and determination of metals and sulfide

UV–visible absorption spectra of reduced form were obtained using an air-tight screw capped cuvette using a Jasco V550 spectrophotometer (Jasco, Hachioji, Japan). EPR spectra were recorded on a X-band spectrometer (model E500, Bruker) equipped with a liquid helium flow cryostat (model ESR900, Oxford). The modulation amplitude was 0.5 mT and microwave was at 20 mW. Metal content of purified L-protein was measured by using an inductively coupled plasma (ICP) analyzer, IRIS (Nippon Jarrell-Ash Co. Ltd., Kyoto, Japan). Wavelengths used for the assay of Co, Cu, Fe, Mn, Mo, Ni, V and Zn were 228.6, 327.3, 271.4, 191.5, 203.8, 341.4, 309.3, and 202.5 nm, respectively. As the standard for each metal, 10, 20, 30 and 40 nM solutions were used (Wako Pure Chemical Industries, Osaka, Japan). Sulfide content was determined according to the protocol [9].

2.6. Oxygen sensitivity

Crude extract containing L-protein (6 × HN) were exposed to air on a piece of Parafilm (Pechiney Plastic Packaging, Chicago, IL). Aliquots were recovered from the drop and the L-protein activity was assayed as described above. L-protein with 6 × HN was purified as above in a buffer without sodium dithionite. The preparation of purified L-protein was exposed to air by mixing with an air bubble in a syringe. Aliquots were recovered from the syringe and mixed with an excess amount of sodium dithionite. L-protein activity was assayed as described above.

3. Results and discussion

3.1. Overexpression and purification of affinity-tag L-proteins in R. capsulatus

To facilitate the purification procedure of L-protein, we constructed two plasmids to overexpress BchL as N-terminal fusion proteins with 6 × HN-tag and Strep-tag under the control of the *puc* promoter (Fig. 1(A)) [2,6]. Both affinity tag BchL proteins were purified by a one-step procedure with affinity columns, to be almost single bands in SDS–PAGE (Fig. 1(B)). The specific activities of purified L-proteins were estimated to be 96 and 100 $nmol_{Chlide}\ min^{-1}\ mg^{-1}_{protein}$ for strep-tag and 6 × HN-tag L-proteins, respectively. Thus, purified Strep-tag and 6 × HN-tag L-proteins were characterized.

3.2. Absorption spectra, EPR spectrum and determination of metals and sulfide of L-protein

Fig. 2(A) shows the UV–visible absorption spectra of reduced and oxidized L-proteins. The reduced L-protein exhibited a broad absorption between 390 and 650 nm with no distinctive peaks (Fig. 2(A), trace a). Upon exposure to air, absorption increased and showed a pronounced shoulder at about 410 nm (Fig. 2(A), trace b). These spectral characteristics were similar to those of nitrogenase Fe proteins from various sources [10,11].

The probable iron–sulfur center present in L-protein was characterized by recording EPR spectra of dithionite-reduced purified L-protein (Fig. 2(B)). L-protein exhibited a rhombic-type signal with g-values of 2.03, 1.94, and 1.86, which are typical of a [4Fe–4S] cluster. The g-values of this signal are almost the same as those in various nitrogenase Fe proteins with slight shift of $g = 2.03$ and 1.86 values [12].

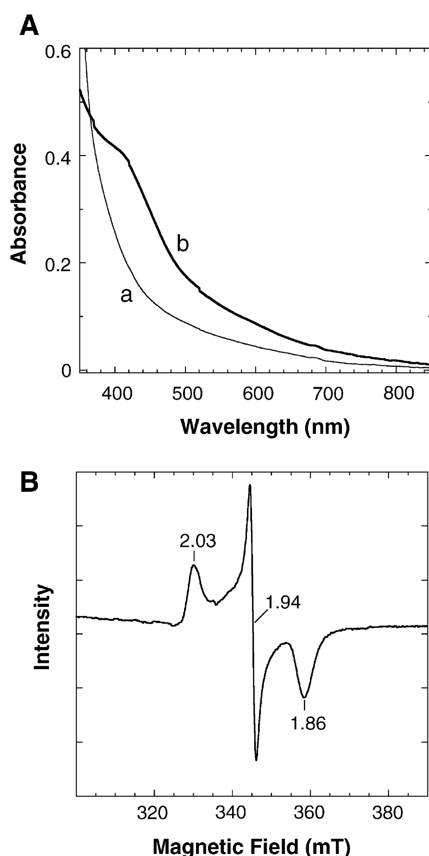

Fig. 2. (A) Absorption spectra of reduced (trace a) and oxidized (trace b) forms of Strep-tag L-protein. (B) EPR spectrum of the reduced L-protein. Purified Strep-tag L-protein was concentrated by ammonium sulfate precipitation (60% saturation). After centrifugation at 260,000 × g for 30 min, L-protein was dissolved in Tris buffer (Tris–HCl (pH 8.0), 10 mM dithithreitol, 10 mM β-mercaptoethanol and 1.7 mM sodium dithionite). EPR spectrum was recorded at 20 K with microwave frequency 9.403 GHz and modulation frequency 100 kHz. Relevant g-values are indicated.

Metal and sulfide of purified L-protein were determined (Table 1). Only iron was reproducibly detected in a significant amount (more than 1 per BchL-dimer) among eight kinds of metal examined (Co, Cu, Fe, Mn, Mo, Ni, V and Zn), while the other metals were not detected (Co and Mo) or very close to the detection limit (Cu, Mn, Ni, V and Zn). The iron content was 2.5 mol per L-protein (BchL-dimer). Sulfide content was also determined to be 3.1 mol per L-protein. The iron and sulfide contents are slightly less than 4.0, which is the theoretical value for iron and sulfide of a [4Fe–4S] cluster, suggesting that the preparation of purified L-protein contained a significant amount of apo-forms of L-protein.

Absorption spectral changes in the redox states and the EPR signal of the reduced form indicated that L-protein has a [4Fe–4S] cluster similar to nitrogenase Fe protein. The homodimeric structure of L-protein ($(BchL)_2$) [6] and the conservation of two Cys residues in BchL (Cys131 and Cys165) are consistent with the presence of the nitrogenase Fe protein-type cluster.

3.3. Oxygen stability of purified L-protein

To address the sensitivity of L-protein to oxygen, we exposed the crude extracts and purified L-protein to air for various periods, and then measured the activity (Fig. 3). L-protein activity of crude extracts diminished with a half-life of about 40 min (Fig. 3(A)), and the activity of purified L-protein was more quickly lost upon the exposure to air with a half-life of about 20 s (Fig. 3(B)). When the same procedures were carried out under an anaerobic environment, L-protein activity of the crude extracts and purified L-protein was kept constant in the time periods we examined. This result indicates that L-protein is very sensitive to oxygen. Taken together, it is suggested that L-protein carries an oxygen-sensitive [4Fe–4S] cluster as well as nitrogenase Fe protein. The half-life of purified L-protein is similar to those of Fe proteins reported [10–15]. The Fe–S cluster of Fe protein is exposed to the solvent and is quickly damaged by oxygen causing irreversible denaturation of Fe protein. The similar oxygen sensitivity of L-protein to Fe protein including the location of Fe–S cluster, suggests that L-protein has an overall structure similar to that of Fe protein.

L-protein activity in crude extracts was much more stable in air than the purified L-protein (Fig. 3). A similar phenomenon is known as 'conformational protection' of nitrogenase in *Azotobacter*. Nitrogenase activity in the crude extracts of *Azotobacter chroococcum* was much more stable to oxygen than purified nitrogenase components. Half-lives of activities of crude extracts and purified components were 24 min and 30 s, respectively [16], which are similar to those of DPOR activity of crude extracts and purified L-protein (Fig. 3). A ferredoxin-like protein with a [2Fe-2S] cluster called FeSII (Shethna) protein mediates the conformational protection [17]. The longer half-life upon oxygen inactivation of L-protein in crude

Table 1
Metal and acid labile sulfur contents of purified L-protein (Strep-tag)

	Co	Cu	Fe	Mn	Mo	Ni	V	Zn	S[a]
L-protein	ND	0.244	2.53	0.131	ND	0.067	0.209	0.031	3.1

Metal and acid labile sulfur contents are shown as molar ratio to L-protein (BchL-dimer). ND: not detected.
[a]Acid labile sulfur content was determined by a colorimetric method [9].

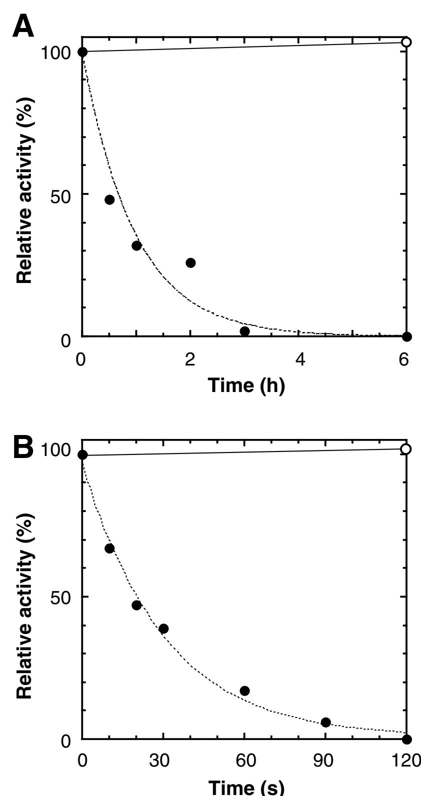

Fig. 3. Oxygen sensitivity of L-protein. Crude extracts containing overexpressed L-protein (6 × HN-tag) (A) and purified L-protein (6 × HN-tag) (B) were exposed to air (solid circles) or to anaerobic atmosphere (in the anaerobic chamber; white circles) for various time periods. After exposure, aliquots were recovered and the L-protein activity was assayed. Theoretical decay curves with half-lives of 40 min and 20 s are shown as dashed lines in panels (A) and (B), respectively.

extracts implies that L-protein of DPOR is also protected from oxygen by a similar protein in the crude extracts. A [2Fe-2S] ferredoxin (*fdxC*, [18]; *fdxD*, [19]) shows the highest similarity to FeSII protein among six ferredoxins in *R. capsulatus*. Which protein(s) is involved in the probable conformational protection for DPOR remains as a future research project.

4. Conclusion

This is the first report demonstrating that the metallocenter of DPOR L-protein is an oxygen-sensitive [4Fe–4S] cluster, which is very similar to the [4Fe–4S] cluster of nitrogenase Fe protein. The amino acid sequence of BchL from *R. capsulatus* shows 29.0% identity to that of NifH from *A. vinelandii*, including the two Cys residues for Fe–S cluster chelation and ATP-binding motif. This relatively low level of sequence identity seems to be sufficient to maintain the structure and function as the ATP-dependent reductase component. The results of this study suggest that the ATP-dependent electron transfer mechanism common to nitrogenase operates in a diverged enzyme DPOR.

Acknowledgements: We thank Akira Watanabe and Etsuko Miyamoto for the metal analysis with ICP. We thank Tatsuo Omata and Kazuki Terauchi for valuable discussions. This work was supported by Grants-in-Aid for Scientific Research (Nos. 14390051 to KI and YF, 15570033 to YF, 13CE2005 and 14COEA2) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] Fujita, Y. and Bauer, C.E. (2003) The light-independent protochlorophyllide reductase: A nitrogenase-like enzyme catalyzing a key reaction for greening in the dark. In: Porphyrin Handbook (Kadish, K.M., Smith, K.M. and Guilard, R., Eds.), Chlorophylls and bilins: Biosynthesis, synthesis, and degradation, vol. 13, pp. 109–156, Academic Press, San Diego.

[2] Nomata, J., Mizoguchi, T., Tamiaki, H. and Fujita, Y. (2006) A second nitrogenase-like enzyme for bacteriochlorophyll biosynthesis: Reconstitution of chlorophyllide *a* reductase with purified X-protein (BchX) and YZ-protein (BchY–BchZ) from *Rhodobacter capsulatus*. J. Biol. Chem. 281, 15021–15028.

[3] Yamazaki, S., Nomata, J., Fujita, Y. (in press) Differential operation of dual protochlorophyllide reductases for chlorophyll biosynthesis in response to environmental oxygen levels in the cyanobacterium *Leptolyngbya boryana*. Plant Physiol.

[4] Igarashi, R.Y. and Seefeldt, L.C. (2003) Nitrogen fixation: The mechanism of the Mo-dependent nitrogenase. Crit. Rev. Biochem. Mol. Biol. 38, 351–381.

[5] Fujita, Y. and Bauer, C.E. (2000) Reconstitution of light-independent protochlorophyllide reductase from purified BchL and BchN–BchB subunits: *In vitro* confirmation of nitrogenase-like features of a bacteriochlorophyll biosynthesis enzyme. J. Biol. Chem. 275, 23583–23588.

[6] Nomata, J., Swem, L.R., Bauer, C.E. and Fujita, Y. (2005) Overexpression and characterization of dark-operative protochlorophyllide reductase from *Rhodobacter capsulatus*. Biochim. Biophys. Acta 1708, 229–237.

[7] Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop II, R.M. and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175–176.

[8] Prentki, P. and Krisch, H.M. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene 29, 303–313.

[9] Timcenko, L. and Kimura, T. (1979) Liberation of labile sulfur from ferredoxins by alkaline zinc reagent: an appraisal of the methylene blue method. Anal. Biochem. 95, 452–457.

[10] Eady, R.R., Smith, B.E., Cook, K.A. and Postgate, J.R. (1972) Nitrogenase of *Klebsiella pneumoniae*: Purification and properties of the component proteins. Biochem. J. 128, 655–675.

[11] Shah, V.K. and Brill, W.J. (1973) Nitrogenase IV. Simple method of purification to homogeneity of nitrogenase components from *Azotobacter vinelandii*. Biochim. Biophys. Acta 305, 445–454.

[12] Eady, R.R. (1980) Isolation and characterization of various nitrogenases. Methods Enzymol. 69, 753–778.

[13] Eady, R.R., Richardson, T.H., Miller, R.W., Hawkins, M. and Lowe, D.J. (1988) The vanadium nitrogenase of *Azotobacter chroococcum*: Purification and properties of the Fe protein. Biochem. J. 256, 189–196.

[14] Whiting, M.J. and Dilworth, M.J. (1974) Legume root nodule nitrogenase: Purification, properties, and studies on its genetic control. Biochim. Biophys. Acta 371, 337–351.

[15] Yates, M.G. and Planqué, K. (1975) Nitrogenase from *Azotobacter vinelandii*: Purification and properties of the component proteins. Eur. J. Biochem. 60, 467–476.

[16] Robson, R.L. (1979) Characterization of an oxygen-stable nitrogenase complex isolated from *Azotobacter chroococcum*. Biochem. J. 181, 569–575.

[17] Moshiri, F., Kim, J.W., Fu, C. and Maier, R.J. (1994) The FeSII protein of *Azotobacter vinelandii* is not essential for aerobic nitrogen fixation, but confers significant protection to oxygen-mediated inactivation of nitrogenase *in vitro* and *in vivo*. Mol. Microbiol. 14, 101–114.

[18] Saeki, K., Suetsugu, Y., Tokuda, K., Miyatake, Y., Young, D.A., Marrs, B.L. and Matsubara, H. (1991) Genetic analysis of functional differences among distinct ferredoxins in *Rhodobacter capsulatus*. J. Biol. Chem. 266, 12889–12895.

[19] Armengaud, J., Meyer, C. and Jouanneau, Y. (1994) Recombinant expression of the *fdxD* gene of *Rhodobacter capsulatus* and characterization of it product, a [2Fe–2S] ferredoxin. Biochem. J. 300, 413–418.