0273-2289/99/77–79/0169/$12.75

Isolation and Analysis of Tetraheme-Bound-Cytochrome from Photosynthetic Reaction Centers of *Rhodopseudomonas viridis*

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Abstract

A tetraheme cytochrome (BCyt*c*) was isolated from the photosynthetic reaction centers (RC) of *Rhodopseudomonas viridis* while maintaining the redox activity. BCyt*c* was removed from the H-subunit-detached RC by polyacrylamide electrophoresis using an alkyl ether sulfate mixed with sodium dodecyl sulfate. Redox titration of BCyt*c* showed a simple one-step redox titration curve and a lowered midpoint potential than that of one in RC. Direct electron transfer between BCyt*c* and electrode surfaces, such as indium tin oxide, was successfully performed, indicating a potential for molecular electronic material.

Abbreviations: BCyt*c*, the bound cytochrome *c*; RC, photosynthetic reaction centers; RC^{-H}, H-subunit-detached RC; AES, sodium polyoxyethylene alkyl ether sulfate; SDS, Sodium dodecyl sulfate; β-OG, *n*-octyl-β-D-glucoside; HNQ, 2-hydroxy-1,4-naphthoquinone; TNTB, tetranitro tetrazolium blue; DAD, diaminodurene; PMS, phenazine methosulfate; ITO, indium tin oxide; SHE, standard hydrogen electrode

Index Entries: Tetraheme cytochrome; surfactant; photosynthetic reaction center; photosynthetic bacterium.

Introduction

Multi-heme cytochromes have a special importance in electron transfer in biological systems. Because of the dense packing of hemes in the molecules, they have unique redox properties. A tetraheme cytochrome,

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cytochrome c_3, isolated from *Desulfovibrio* sp., has been intensively investigated. It shows high electron conductivity because of the four closely packed hemes in the polypeptide moiety *(1–3)*.

Another type of tetraheme cytochrome was found in bacterial photosynthetic reaction centers (RCs) from various photosynthetic purple bacteria, such as *Rhodopseudomonas viridis*, *Rhodospirillum molischianum*, *Rubrivivax gelatinosus*, and *Chromatium vinosum (4)*. The RC is located in the chromatophore membrane and plays a main role in the photosynthesis of photo-excited charge separation, which starts from excitation of the bacteriochlorophyll special pair. The three-dimensional structure of the RC from *Rps. viridis* was clarified by Deisenhofer *(5)*. The RC-bound cytochrome (BCyt*c*) has a unique structure with four linearly aligned hemes in the molecule. The first redox voltammetry titration of a chromatophore membrane from *Rhodobacter sphaeroides* was reported by Seibert et al. *(6)*. The redox properties of BCyt*c* from *Rps. viridis* were also determined by several groups *(7–11)*. The four hemes in the molecule form two pairs, one with high potential and the other with low potential. The sequence order of the hemes from the far side to the near side with the special pair is *c*-554 (–60 mV), *c*-556 (310 mV), *c*-552 (20 mV), and *c*-559 (380 mV) *(4,8)*. This structure is especially useful to donate electrons to the special pair. One potential use for the linearly aligned hemes is as molecular wires. We reported the light-induced electrical responses of the dried chromatophore film of the photosynthetic bacteria, *Rps. viridis* and *Rb. sphaeroides* whose RC does not contain a BCyt*c (12)*. The chromatophore of *Rps. viridis* showed a greater current response than that of *Rb. sphaeroides (12)*. This suggests that BCyt*c* is applicable as an prominent conductive material in such biochemical devices. However, no method has been developed to isolate the BCyt*c* molecules of *Rps. viridis*, because BCyt*c* is a part of the membrane protein that is tightly bound through a large contact area to the ML complex and anchored into the chromatophore membrane by two lipids from the N-terminus *(13)*. We have tried in vain various surfactant chemicals and enzymatic treatments using chaotropic ion, lipase, or proteinase to isolate the BCyt*c* from the *Rps. viridis* RC.

Previously we have found that the H-subunit could be separated from the RC by preparative polyacrylamide gel electrophoresis (PAGE) using sodium polyoxyethylene alkyl ether sulfate (AES) and demonstrated that the photosynthetic ability of RC was maintained after the preparation *(14,15)*. In this work, we further improved the method for the isolation of BCyt*c* from RC. This is the first report describing the isolation and the analysis of the electrochemical characteristic of the tetraheme cytochrome *c*-subunit molecule from the *Rps. viridis* RC.

Materials and Methods

Surfactants

Sodium dodecyl sulfate (SDS) was purchased from Nacarai Tesque Co. Ltd. (Kyoto, Japan), two AESs, NES-203-27 [$RO(CH_2CH_2O)_3SO_3Na$,

R = $CH_3(CH_2)_{11}$ or $CH_3(CH_2)_{12}$] and SBL-4N [$CH_3(CH_2)_{11}O(CH_2CH_2O)_4SO_3Na$], were purchased from Nikko Chemicals Co. Ltd. (Tokyo, Japan), and *n*-octyl-β-D-glucoside (β-OG) was purchased from Dojindo Laboratories (Kumamoto, Japan).

Bacterial Culture and H-Subunit-Depleted RC Preparation

Rps. viridis ATCC 19567 was cultured by ASY medium *(16)*. Chromatophore isolation from bacterial cells and RC purification from chromatophore were done as previously reported *(17)*. H-subunit-depleted RC (RC^{-H}) was prepared by PAGE using the surfactant NES-203-27. The details of these methods are described elsewhere *(14)*.

*Isolation of BCyt*c

An RC^{-H} solution of 5 mL (17 μ*M*) dissolved in 10 m*M* Tris-HCl buffer, pH 8.2, was mixed with 5 mL of the mixed surfactant of 5% (w/v) SDS and 5% (w/v) SBL-4N. Electrophoresis was done according to the method of Laemmli *(18)*. Ten mL of the mixture was added to 2 mL of glycerol and subjected to PAGE using an electrode buffer containing 25 m*M* Tris, 200 m*M* glycine, 0.05% (w/v) SDS, and 0.05% (w/v) SBL-4N. Isolation was also done by using 10% (w/v) SDS solution and an electrophoresis buffer containing 0.1% (w/v) SDS. The concentration of acrylamide was 12% for separation and 4% for stacking gels. The gel-plate size was 150-mm wide, 100-mm long, and 5-mm thick. The electrophoresis was done with 20 mA/gel for about 18 h at 4°C. A red band containing BCyt*c* was cut from the gel. The gel was ground using a homogenizer and the protein was extracted with a 10 m*M* Tris-HCl buffer, pH 8.2, containing 0.5% (w/v) β-OG. The gel pieces were removed by filtration and the filtrated solution was concentrated on an Amicon PM-30 ultrafiltration membrane (Amicon, Beverly, MA). The protein concentration was measured by the Bradford method *(19)*.

Analytical SDS-PAGE and Heme Staining

The purity of the cytochrome subunit was assayed by SDS-PAGE, and 27 pmol of BCyt*c* was loaded onto two gels. One gel was stained by Coomassie Brilliant Blue R-250, and the other by heme-staining, according to the method of Thomas *(20)*.

Spectral Analysis

The spectra of BCyt*c* were measured using two spectrophotometers, U-3500 (Hitachi, Tokyo, Japan) and UV-1601 (Shimadzu, Kyoto, Japan). Every spectra were stored and mathematically analyzed using computer software, Microcal™ Origin™ (Microcal Software, Inc., Northampton, MA): the characteristics and the absorption coefficients of the hemes were estimated from a spectrum of the reduced BCyt*c* minus one of the oxidized BCyt*c*. Dithionite was used for the reduction of BCyt*c*.

*Redox Titration of BCyt*c

The redox titration was done according to the method described by Dracheva et al. *(8)* using a sealed cuvet under anaerobic conditions, which were achieved by argon ventilation. The basic solution for redox titration was a 10 m*M* MOPS buffer, pH 8.0, containing 100 m*M* KCl and 0.05% β-OG. Mediators of 50 μ*M* each of HNQ, TNTB, DAD, and PMS were added. The final concentration was 1.5 μ*M* each of BCyt*c* and RC^{-H}. The oxidized state was adjusted using a 150 μ*M* potassium ferricyanide. Dithionite was used for reduction. The redox potential was measured *in situ* by a pH meter, HM-30S, with the ORP electrode, PTS-5011C (TOA Electronics Ltd., Tokyo, Japan). The state showing the maximum absorbance of α bands of BCyt*c* was assumed to correspond to the completely reduced state of BCyt*c*.

Cyclic Voltammetry

Cyclic voltammetry was done in a three-electrode system using an electrochemical analyzer, BAS 100B (BAS, Tokyo, Japan). The three electrodes were indium tin oxide (ITO), Ag/AgCl, and platinum (the working, reference, and counter electrodes, respectively). In the voltammogram measurements, the effective area of the ITO electrode was 150 mm^2, and the solution for was 100 m*M* potassium phosphate buffer containing 300 m*M* NaCl and 40 m*M* BCyt*c*. The measurement was done under anaerobic conditions using a sealed vessel in a manner similar to the redox titration. Dithionite was used for the adjustment of the redox potential of the solution, and the redox potential was measured as previously described.

Results and Discussion

*Purification of BCyt*c

The isolation of membrane proteins and the separation of their subunits require surfactants, because membrane proteins are usually highly hydrophobic. However, powerful surfactants, such as SDS, denature proteins and the subunits, whereas mild surfactants, such as β-OG, can not dissociate subunits of such membrane proteins. Surfactants must be able to dissociate subunits with minimum denaturation. Takagi et al. *(21–24)* studied SDS analogs for their milder effect on proteins but sufficient solubilization ability. They reported that AESs are milder than SDS but have comparable solubilization capability as SDS. AESs are effective in isolating the subunits of RCs. For RC, SDS completely decomposes the protein into subunits with denaturation. One type of AES, NES-203-27, removes H-subunits from the RC without reducing the activity of RC *(14)*. However, with this AES alone, BCyt*c* could not be isolated from the RC.

In this work, we examined mixtures of AES and SDS for possible enhancement in solubilization by AES. One of the AES, SBL-4N was used because it exhibits slightly better solubilization properties than NES-203-27. RC^{-H} was used as the starting material to avoid contamination of the

Fig. 1. Picture of the preparative PAGE. Gel without staining. RC^{-H} was loaded using a 1:1 mixture of SBL-4N and SDS. The arrow indicates a red-colored BCyt*c* band.

H-subunit. Figure 1 shows a separation pattern of the preparative SBL-4N/SDS PAGE for the BCyt*c* isolation. We observed a red band of the isolated BCyt*c*, indicated by the arrow. Although M- and L-subunits were separated from the cytochrome band, these bands did not retain chromophores (see the lower thick band in Fig. 1). RC^{-H} remained above the red band after electrophoresis. Using SDS only, the red band was thicker and the band of remaining RC^{-H} was thinner (data not shown). This demonstrates the strong solubilization by SDS compared with that of the AES-SDS mixture. The red band in the preparative PAGE was cut out and the cytochrome subunit was extracted. The sample was dissolved in 10 m*M* Tris-HCl buffer, pH 8.2, containing 0.5% β-OG and concentrated up to 20 μ*M* of protein concentration.

*Analytical Electrophoresis of BCyt*c

Analytical SDS-PAGE (Fig. 2) shows the purity of BCyt*c* isolated by the preparative PAGE. Only the 40 kDa band could be observed in the isolated sample using the SBL-4N/SDS mixture (lane 1), whose size corresponds to the BCyt*c* band of RC^{-H} in lane 3. The heme-staining protein with the same apparent molecular size as the Coomassie staining band was observed (lane 4). A wide but weak band above the main band in lane 4 should contain a highly aggregated complex of proteins formed in the isolation process. These results indicate that BCyt*c* was successfully purified by the SBL-4N/SDS-PAGE preparation. No significant difference was observed in the sample isolated using SDS only (lanes 2 and 5) compared with the sample SBL-4N/SDS mixture.

*Spectral Analysis of BCyt*c

The spectra of the isolated BCyt*c*, using the detergent mixture, were measured at sufficiently reduced and oxidized conditions (Fig. 3A). Entire autoxidation of BCyt*c* occurred in the buffer solution before addition of

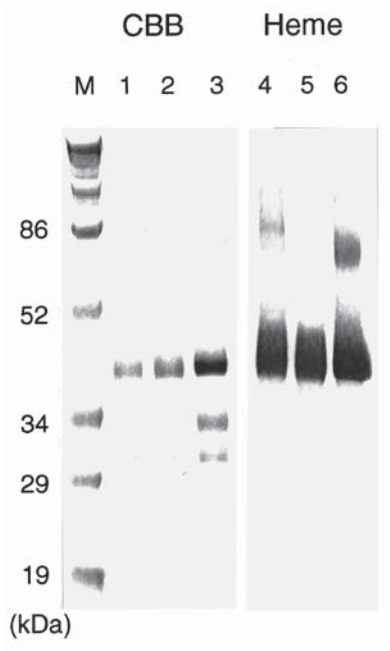

Fig. 2. SDS-PAGE analysis and heme staining of the purified BCyt*c*. Lane M shows the molecular weight marker. The isolated BCyt*c* using an SBL-4N/SDS mixture was loaded in lanes 1 and 4. The isolated BCyt*c* using SDS only was loaded in lanes 2 and 5. RC^{-H} was loaded in lanes 3 and 6, 27 pmol of protein was loaded in each lane.

the reductant. The red shift of the Soret band from 408 to 418 nm was caused by the addition of dithionite, similar to the shift seen for other cytochromes. The Q bands, β band at 523 nm, and α band at 552 nm also appeared in the sufficiently reduced state. These absorption peaks indicate the retained redox ability of BCyt*c*, even after the stringent isolation step. The absorptional spectra of the 1 μ*M* SDS-isolated material (same concentration as the mixture-isolate) were shown in Fig. 3B. Although the curve shape of reduced and oxidized spectra of the SDS-isolate were very similar to those of the mixture-isolate, the peak heights were obviously different. The lower absorbency of the SDS-isolate would be owing to denaturation by SDS.

Reduced–oxidized spectrum of the isolated BCyt*c* is given in Fig. 4, focusing on the region of the α-absorption band. A narrow symmetrical α band was seen only at 552 nm. We tried to extract four individual peaks corresponding to the four hemes from the α band of BCyt*c* at 552 nm using the method of Drachieva et al. *(8)* without success (data not shown). It indicates that the peak at 552 nm consists of the sum of the all hemes in the isolated BCyt*c*. A $\Delta\varepsilon_{red\text{-}ox}$ value of the 552 nm band estimated from the peak height of subtracted spectrum was 78.8 $mM^{-1} \cdot cm^{-1}$, which is over two times higher than the value of $\Delta\varepsilon_{red\text{-}ox}$ for *c*-552 from the native RC, 34 $mM^{-1} \cdot cm^{-1}$ *(8)*. This suggests that the α-band shift occurred in the isolation step and that some peaks coalesce in the 552-nm band. In contrast, the $\Delta\varepsilon_{red\text{-}ox}$ value from the

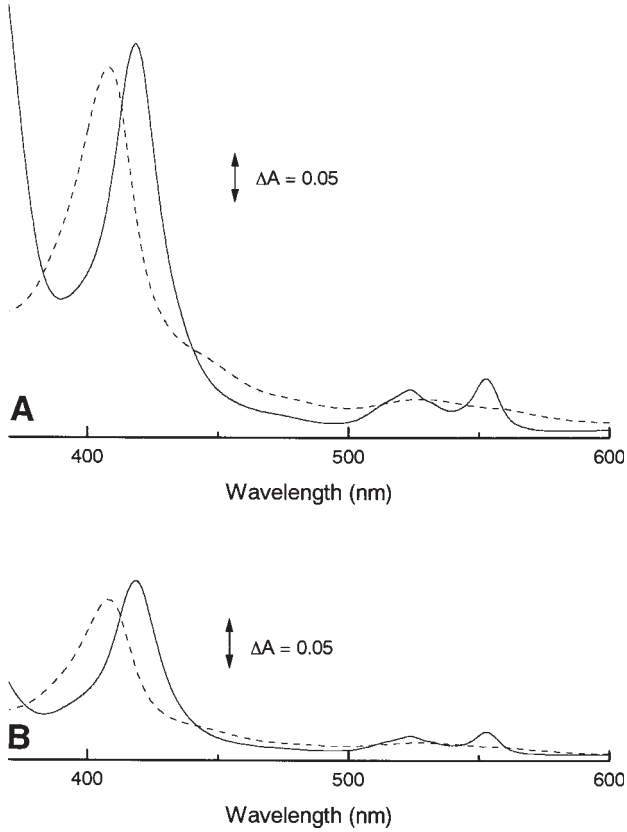

Fig. 3. Spectra of the isolated BCyt*c*. Solid and dashed lines show the spectrum of the reduced and oxidized BCyt*c*, respectively. Spectra of 1 μ*M* of BCyt*c* isolated by using SBL-4N/SDS mixture **(A)**, spectra of 1 μ*M* of only SDS **(B)**.

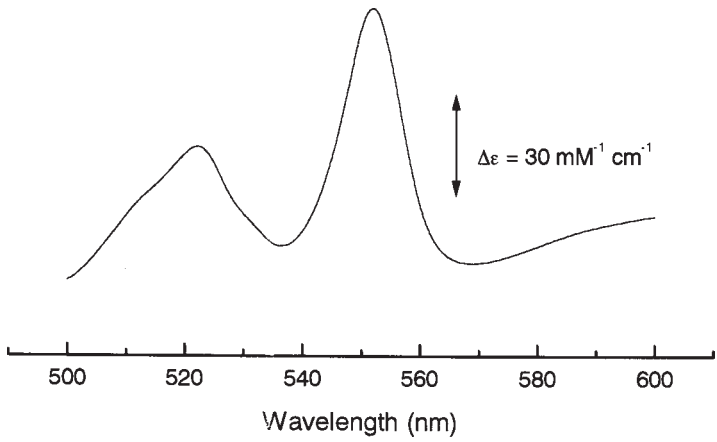

Fig. 4. Subtracted α-band spectrum of BCyt*c* The figure shows the spectrum produced from the simple subtraction of the entirely reduced state minus the oxidized state.

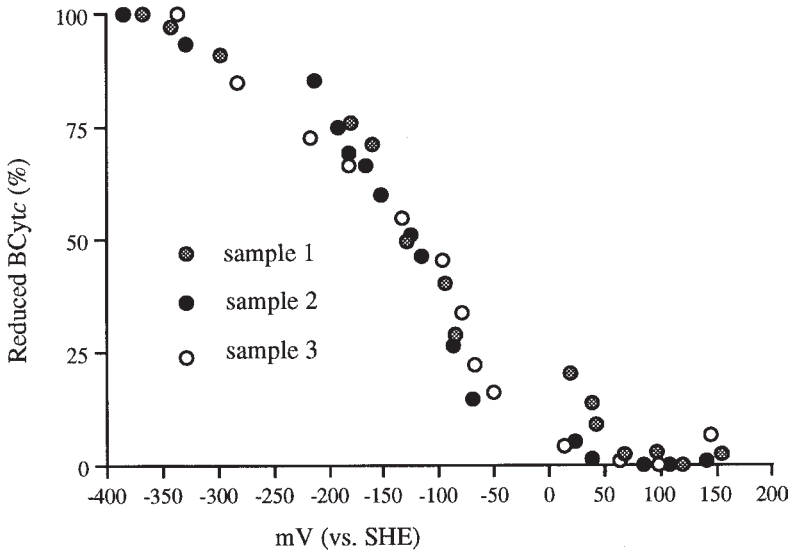

Fig. 5. Redox titration curve of BCyt*c*. The results of three independent measurements are represented (see Materials and Methods section for detail).

SDS isolation, 33.1 $mM^{-1} \cdot cm^{-1}$, was less than half that from the SBL-4N/SDS sample. This indicates that SBL-4N counteracts the strong effect of denaturation by SDS in the isolation process.

*Redox Characteristics of BCyt*c

The state showing the maximum absorbance at 552 nm was assumed to correspond to the completely reduced state of the isolated BCyt*c* in the redox titration. The titration for three different isolates is shown in Fig. 5. The midpoint of the redox potential is approx –120 mV (relative to SHE), which is 100 mV lower than E_m = –20 mV of the low-potential heme pair (*c*-554 and *c*-552) *(8)*. The α absorption band and E_m shift of BCyt*c* indicate that the heme environment in the isolated molecule was denatured by the surfactant treatment. Although the atomic absorption spectrophotometric analysis for iron content of the isolated BCyt*c* indicated that the entire four-heme-group structure was retained (data not shown), the electrochemical characteristics of the high potential hemes (*c*-556 and *c*-559) were apparently lost in the isolated BCyt*c*. The position of the highest potential *c*-559 was closest to the exposed BCyt*c* surface, achieved by detaching from the ML subunit, and it appears that the cytochrome c_2 binding site is near the *c*-556 heme *(25)*. Presumably, surfactants easily contact the sites closest to the high potential hemes.

*Cyclic Voltammogram of BCyt*c

In order to evaluate the BCyt*c* applicability as an electrochemical device, we investigated the direct electron transfer between the protein and

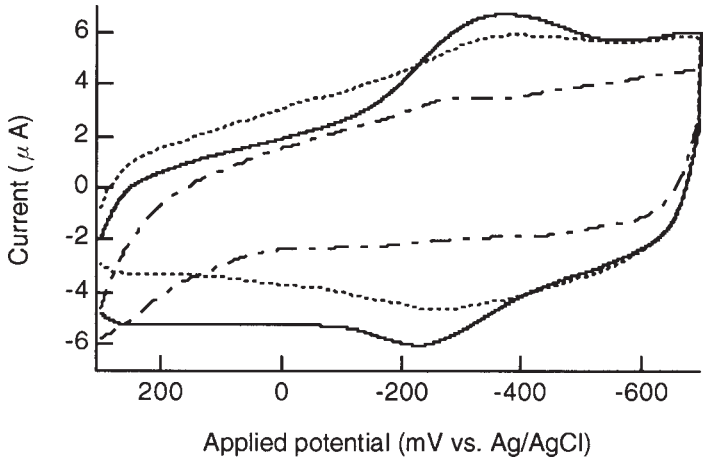

Fig. 6. Cyclic voltammogram of BCyt*c* using ITO as a working electrode. Solid and dotted lines show the voltammograms of the BCyt*c* measured at –100 mV and –40 mV (vs SHE), respectively. The dashed line shows a blank control measurement at –130 mV (vs SHE), without BCyt*c*.

a bare ITO electrode in the aqueous system. Owing to the low-redox potential of BCyt*c* deaeration was needed to prevent the autonomous oxidation of BCyt*c* by oxygen. Argon bubbling or flush are used in the cyclic voltammetry of cytochrome c_3 *(26,27)*. However, this technique was not able to be applied because the sample contained detergents that created many bubbles. Therefore we used the chemical reductant to deaerate the sample solution. The redox potential of the sample solution was carefully adjusted at –40 and –100 mV (vs SHE), higher than the E_m value from the titration, to prevent an excess reduction. Figure 6 shows the cyclic voltammogram of BCyt*c* under the low redox state. The blank control (without BCyt*c*) at –130 mV demonstrated that the reducing chemical does not generate current directly in the voltammetry. Cathodic and anodic current peaks were clearly seen when the redox state was poised at –100 mV by the reductant. The usual peak analysis of cyclic voltammetry could not be performed because the chemical reductant contribution is not negligible. However this result demonstrates the ability of BCyt*c* to transfer electrons reversibly with the bare ITO electrode, even after stringent isolation.

Conclusion

This work provided a new material for the study on electronic devices. Because BCyt*c* has a moderate redox potential, the electronic functions can be performed in a usual redox condition at room temperature. On the other hand, the midpoint of the redox potential of another tetraheme cytochrome, cytochrome c_3 was as low as –300 mV (vs SHE) at 25°C *(28,29)*. The cyclic voltammograms of cytochrome c_3 also have indicated the electron transfer with electrodes at approximately –300 mV *(27,30,31)*. Such a low-redox

potential makes conditions very difficult for the reaction of the electron mediation under aerobic condition or coupling with high potential enzymes. The new type of multi-heme cytochrome isolated in this work should be of use to investigate the electron transfer *in vitro*. Owing to the special arrangement of the hemes in the molecule, molecular handling to organize BCyt*c* in an order could provide materials with novel electronic characteristics. Also, molecular-level combination with other redox proteins or electroconductive materials could enable the development of molecular devices.

Acknowledgments

We thank Dr. Alina Dudkowiak of our laboratory for the spectral analysis. This work was supported in part by the Bionic Design project of the National Institute for Interdisciplinary Research, and in part by the project for "Research and Development of Protein Molecular Assembly," sponsored by the Agency of Industrial Science and Technology, Ministry of International Trade and Industry.

References

1. Higuchi, Y., Kusunoki, M., Matsuura, Y., Yasuoka, N., and Kakudo, M. (1984), *J. Mol. Biol.* **172,** 109–139.
2. Pierrot, M., Haser, R., Frey, M., Payan, F., and Astier, J. (1982), *J. Biol. Chem.* **257,** 14,341–14,348.
3. Haser, M., Pierrot, M., Frey, M., Payan, F., Astier, J. P., Bruschi, M., and Gall, L. (1979), *Nature* **282,** 806–810.
4. Nitschke, W. and Dracheva, S. M. (1995), in *Anoxygenic photosynthetic bacteria,* vol. 2, Blankenship, R. E., Madigan, M. T., and Bauer, C. E. (eds.), Kluwer, Dordrecht, pp. 775–805.
5. Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985), *Nature* **318,** 618–624.
6. Seibert, M. and Kendall-Tobais, M. W. (1982), *Biochim. Biophys. Acta* **681,** 504–511.
7. Alegria, G. and Dutton, P. L. (1991), *Biochim. Biophys. Acta.* **1057,** 258–272.
8. Dracheva, S. M., Drachev, L. A., Konstantinov, A. A., Semenov, A. Y., Skulachev, V. P., Arutjunjan, A. M., Shuvalov, V. A., and Zaberezhnaya, S. M. (1988), *Eur. J. Biochem.* **171,** 253–264.
9. Fritz, F., Moss, D. A., and Mantele, W. (1992), *FEBS Lett.* **297,** 167–170.
10. Fritzsch, G., Buchanan, S., and Michel, H. (1989), *Biochim. Biophys. Acta.* **977,** 157–162.
11. Nitschke, W. and Rutherford, A. W. (1989), *Biochem.* **28,** 3161–3168.
12. Majima, T., Miyake, J., Hara, M., Ajiki, S., Sugino, H., and Toyotama, H. (1989), *Thin Solid Films* **180,** 85–88.
13. Weyer, K. A., Schafer, W., Lottspeich, F., and Michel, H. (1987), *Biochemistry* **26,** 2909–2914.
14. Miyake, J., Hara, M., Asada, Y., Morimoto, Y., and Shirai, M. (1998), *Electrophoresis* **19,** 319–322.
15. Hara, M., Kaneko, T., Nakamura, C., Asada, Y., and Miyake, J. (1998), *Biochim. Biophys. Acta* **1363,** 199–208.
16. Miyake, J. and Kawamura, S. (1987), *Int. J. Hydrogen Energy* **12,** 147–149.
17. Jacob, J. S. and Miller, K. R. (1983), *Arch. Biochem. Biophys.* **223,** 282–290.
18. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
19. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
20. Thomas, P. E., Ryan, D., and Levin, W. (1976), *Anal. Biochem.* **75,** 168–176.

21. Ohbu, K., Jona, J., Mizushima, N., and Kashiwa, I. (1980), *Yukagaku* **29,** 866–871.
22. Takagi, T. (1988), *Yukagaku* **37,** 402–407.
23. Takagi, T. (1991), *Adv. Electrophoresis* **41,** 391–406.
24. Koide, M., Fukuda, M., Ohbu, K., Watanabe, Y., Hayashi, Y., and Takagi, T. (1987), *Anal. Biochem.* **164,** 150–155.
25. Meyer, T. E., Bartsch, R. G., Cusanovich, M. A., and Tollin, G. (1993), *Biochem.* **32,** 4719–4726.
26. Sagara, T., Nakajima, S., Akutsu, H., and Niki, K. (1991), *J. Electroanal. Chem.* **297,** 271–282.
27. Hagen, W. R. (1989), *Eur. J. Biochem.* **182,** 523–530.
28. Niki, K., Kawasaki, K., Nishimura, N., Higuchi, Y., Yasuoka, N., and Kakudo, M. (1984), *J. Electroanal. Chem.* **168,** 275–286.
29. Fan, K., Akutsu, H., Kyogoku, Y., and Niki, K. (1990), *Biochemistry* **29,** 2257–2263.
30. Niki, K., Yagi, T., Inokuchi, H., and Kimura, K. (1977), *J. Electrochem. Soc.* **124,** 1889–1891.
31. Moreno, C., Campos, A., Teixeira, M., Legall, J., Montenegro, M. I., Moura, I., Dijk, C. V., and Moura, G. J. (1991), *Eur. J. Biochem.* **202,** 385–393.