Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl19

Cytochrome b_{562}: Electroactive Protein for Bioelectronics

Hiroyuki Sasabe [a], Taiji Furuno [a], Kazutoshi Kobayashi [a], Yukio Kobayashi [a] & Teruyuki Nagamune [a]
[a] Frontier Research Program, The Institute of Physical & Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama, 351-01, Japan
Version of record first published: 23 Sep 2006.

To cite this article: Hiroyuki Sasabe , Taiji Furuno , Kazutoshi Kobayashi , Yukio Kobayashi & Teruyuki Nagamune (1994): Cytochrome b_{562}: Electroactive Protein for Bioelectronics, Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals, 255:1, 271-278

To link to this article: http://dx.doi.org/10.1080/10587259408029798

Mol. Cryst. Liq. Cryst. 1994, Vol. 255, pp. 271–278
Reprints available directly from the publisher
Photocopying permitted by license only

Printed in the United States of America

CYTOCHROME b_{562}: ELECTROACTIVE PROTEIN FOR BIOELECTRONICS

HIROYUKI SASABE, TAIJI FURUNO, KAZUTOSHI KOBAYASHI, YUKIO KOBAYASHI AND TERUYUKI NAGAMUNE
Frontier Research Program, The Institute of Physical & Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-01, Japan

Abstract To develop bioelectronic devices and/or the structural analyses of proteins, two-dimensional crystallization of proteins is required. Heme proteins are particularly interesting due to electron transfer characteristics associated with the porphyrin-based prosthetic group. Cytochrome b_{562} has been chosen as a model protein for the fabrication of two-dimensional crystals. Three approaches in making two-dimensional crystals of cytochrome b_{562} are presented here: (1) the formation of monomolecular layer of cytochrome b_{562} reconstituted with alkylated heme, (2) the control of surface charge density of cytochrome b_{562} and (3) the enhancement of thermal stability both by means of protein engineering.

INTRODUCTION

For the realization of bioelectronic devices, most critical step would be the development of general methods for controlling the spatial arrangement and orientation of proteins to form a highly-ordered state, *i.e.*, two-dimensional crystal. For structural analyses, the availability of two-dimensional crystals of biological macromolecules also offers many advantages. We have investigated different kinds of proteins for two-dimensional crystallization, that is, spherical ferritin and tetrameric catalase monolayer, hexagonal packing of bacteriorhodopsin trimers, square lattice packing of nitrile hydratase dimers, and cytochrome b_{562}. Among them cytochrome b_{562} (cyt-b_{562}) has been chosen in this study as a model protein for the fabrication of two-dimensional crystals. Cyt-b_{562} is a small (M_W=12000) water-soluble protein found in *Escherichia coli*, consisting of 106 amino acid residues[1,2] and classified as an electron transfer protein. The structure of the *Escherichia coli* ferricytochrome b_{562} has been defined by X-ray crystallography to a resolution of 2.5 Å [3]. X-ray crystallographic studies revealed that ferricytochrome

b_{562} consists of four α-helices. The heme, protoporphyrin IX, is noncovalently bound and ligated to the polypeptide chain through methionine 7 on the N-terminal helix and histidine 102 on the C-terminal helix. As shown in Figure 1, cyt-b_{562} has a pair of hair-pins whose arms consist of two α-helices, and the heme sits in between these two hair-pins.

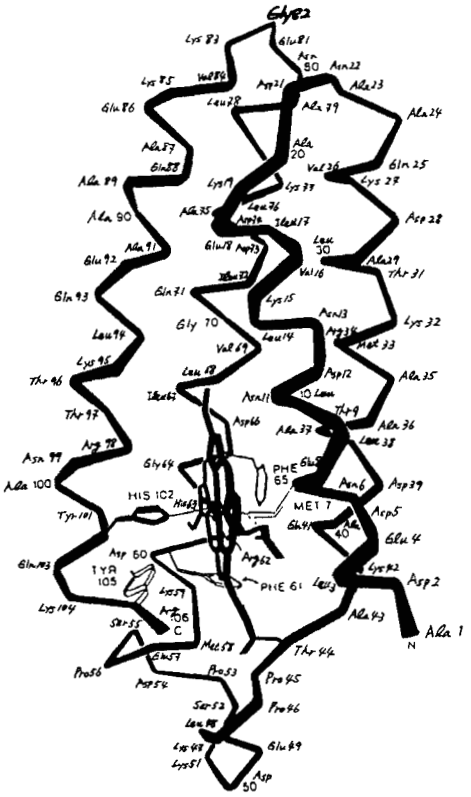

Figure 1. Wire structure of cytochrome b_{562}.

Three approaches are presented in this paper in making two-dimensional crystals of cyt-b_{562}. One is the formation of monomolecular layer of cyt-b_{562} reconstituted with alkylated heme. When crystallizing molecules in two dimension, general method utilized is the Langmuir-Blodgett (LB) technique. If the protein molecules have amphiphilic property at air-water interface, the protein solution can be spread directly onto the water surface. As mentioned, the heme prosthetic group of cyt-b_{562} is not covalently bound to the protein, and can therefore be removed and replaced *in vitro* by other prosthetic groups such as modified porphyrin. As a model case, we synthesized

protoheme IX having long alkyl chain, and reconstituted apocyt-b_{562} with the modified protoheme IX. This would give cyt-b_{562} an amphiphilic property. Second approach is to control the surface charge distribution of cyt-b_{562}, especially the intramolecular charge separation on average. We have developed a novel technique to control the molecular orientation of protein during the adsorption to the charged polypeptide monolayer at the air/water interface using LB technique[4]. This is quite successful for the charged protein molecules such as ferritin and catalase. The third approach is to increase the stability of proteins by protein engineering. When crystallizing proteins at the interface, most proteins denature due to surface pressure effects. There are several techniques to overcome this problem. The enhancement of structural stability of the protein by site-directed mutagenesis would be an approach to prevent denaturation. An effective approach to make a thermally stable mutant protein would be an introduction of disulfide bridge formed by two cysteine residues[5,6] at the top of hair-pins. We made therefore a mutant protein having both asparagine 22 (Asn22) and glycine 82 (Gly82) changed to cysteines. These positions were calculated to be suitable for disulfide bridge formation by means of computer simulation[7].

FORMATION OF MONOMOLECULARLAYER OF ALKYLATED Cyt-b_{562}

Sample Preparation: Cyt-b_{562} was obtained from *Escherichia coli* strain TB-1 harboring pNS 207 plasmid grown in LB subphase and purified as previously described[8] with some modifications. The protein used in this study had an absorption ratio of 6.1 for A_{418}/A_{280} in the oxidized state. The apoprotein was prepared at 4°C using the butanone extraction method[9]. The apoprotein solution was dialyzed against pure water. The modified protoheme IX (stearylated protoheme IX in this study) was obtained from the reaction of protoheme IX with stearylamine in pyridine at 4°C, in the presence of dicyclohexylcarbodiimide.

Reconstitution of Apocytochrome b_{562} with Alkylated Heme: The reconstitution of apocyt-b_{562} with protoheme IX can be achieved by a reaction in alkaline pH buffer[10]. However, since stearylated protoheme is insoluble in water, the mixture of dimethylsulfoxide and water was used as a solvent for reconstitution reaction. The effect of solvent composition of dimethylsulfoxide and water on the reconstitution reaction was examined. As a result, the reconstitution reaction was successful when the dimethylsulfoxide content was 55-65%. The effect of the temperature on the reconstitution reaction was also examined and it is found that the

lower the temperature of reconstitution reaction, the higher the fraction of the reconstitution. From the time evolution experiment of the reconstitution reaction monitored by the Soret band at 418 nm, it was found that the reconstitution reaction takes 30-40 minutes to complete, which is a rather slow reaction.

Surface Property of Modified Cyt-b_{562}: Figure 2 shows the π-A isotherms of stearylated holocytochrome b_{562} (st-cyt-b_{562}) spread on 0.25 mM $CdCl_2$, pH 7.7, at

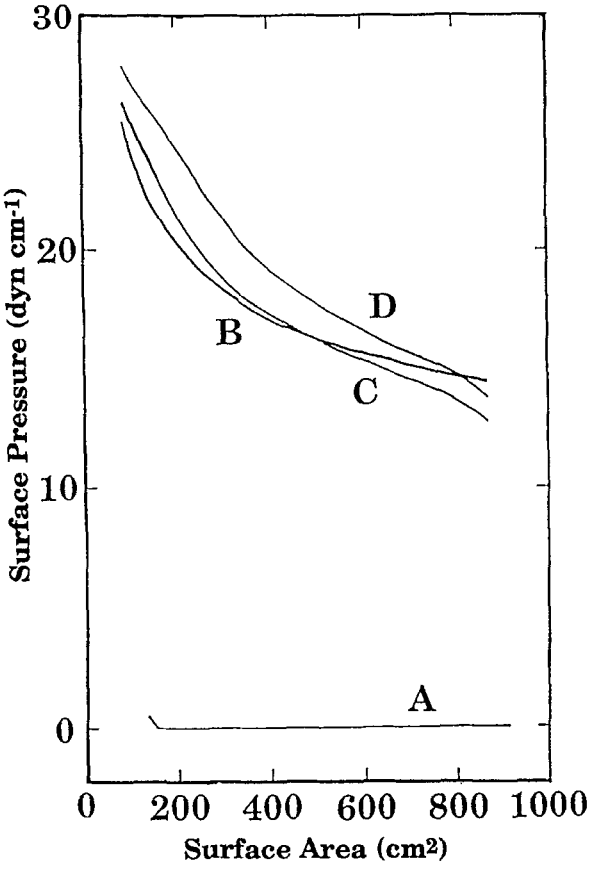

Figure 2. π-A isotherms on 0.25 mM CdCl2, pH 7.7. A: native b_{562}, B: st-cyt-b_{562} in pure water, C: st-cyt-b_{562} in pure water and DMF, D: st-cyt-b_{562} in pure water and isopropyl alcohol.

21°C. St-cyt-b_{562} was prepared in reference to the above results, namely, dimethylsulfoxide content 63%, temperature 10°C, reconstitution time 40 minutes. Generally, it is difficult to spread the protein solution directly onto a buffer surface. Therefore, we utilized dimethylformamide or isopropyl alcohol to aid the procedure. In Figure 2, curve A shows the isotherm of native b_{562} and curve B shows that of stearylated

holocyt-b_{562}. It is clear that curve B shows surface-active property. Similar results were obtained from curves C and D with the addition of dimethylformamide and isopropyl alcohol, respectively. Thus, it is concluded that the presence of organic solvent is not necessary in spreading stearylated holocyt-b_{562} onto a buffer surface. The surface-pressure of *ca.* 26 dyn cm^{-1} given in Figure 2 is thought to be the value inherent to stearylated holocyt-b_{562}. After the compression, the surface pressure decreased gradually and showed a stable value of *ca.* 20 dyn cm^{-1} (data not shown), which indicates that the stearylated holocyt-b_{562} forms a stable monomolecular layer on the buffer surface.

MODIFICATION OF SURFACE CHARGE DISTRIBUTION OF Cyt-b_{562}

By using the computer graphics, the positive and negative charge distribution on the surface of cytchrome b_{562} has been visualized. In order to distribute positive and negative charges unevenly, that is, the one side is positive and the other negative, the point mutation of Asp73 to lysine (Lys) is suggested. (Unfortunately, the figure of charge distribution is not available in a black-and-white picture.) This kind of polarization helps the electrostatic interaction between the protein molecules and charged monolayer formed at the air/water interface to obtain a well-aligned protein monolayer. The site-directed mutagenesis of Asp73 to Lys is now on going. As mentioned in Introduction, we have already succeeded in controlling the molecular orientation of ferritin and catalase by adsorbing electrostatically on to the charged polypeptide (*e.g.*, polybenzylhistidine: PBLH) monolayer. Therefore we can expect the orientational adsorption of charge controlled cyt-b_{562} to the PBLH monolayer.

THERMAL STABILITY OF NATIVE AND MUTANT Cyt-b_{562}

Design of Mutants: The stability of a protein can be increased in several ways: i) by selecting amino acid substitutions to decrease a backbone configurational entropy of unfolding, ii) by introducing disulfide bonds into a protein molecule, iii) by replacing a poor helix-forming residue within an α-helix with a good helix former, iv) by increasing hydrophobicity of a protein core, and so on. On the basis of these strategies, an attempt was made to enhance the thermal stability of cyt-b_{562} by site-directed mutation, *i.e.*, a substitution of glycine residue with alanine or an introduction of disulfide bond at a site consistent with the known three-dimensional structure.

There are three glycine residues (Gly64, Gly70, Gly82) in cyt-b_{562}. The substitution of glycine was chosen so as to avoid the introduction of unfavorable steric interaction in the mutant protein. Inspection of these glycine sites using computer modelling suggested that the residue Gly64 could potentially accommodate a β-carbon without interfering with neighboring atoms. Since Gly64 is located within an α-helix and is buried in the protein interior, the replacement of glycine (poor helix former, low residue hydrophobicity) with alanine (good helix former, high residue hydrophobicity) was expected to enhance thermal stability of the engineered protein by combined stabilization effects of iii) and iv). In order to introduce a single disulfide bond into cyt-b_{562}, the suitable positions to be replaced with cysteine residues were again estimated by computer modelling of mutant protein, and consequently the residues Asn22 and Gly82 were chosen (top of the hair-pins). The disulfide bridge between 22/82 residues is expected to stabilize the wagging of two hair-pins of cyto-b_{562}.

Comparison of Thermal Stabilities between Native and Mutant Proteins: A site-directed mutagenesis was carried out to produce mutants, G64A (Gly64 to alanine (Ala)) and A22C/G82C (Asn22 to cysteine, Gly82 to cysteine). The thermal

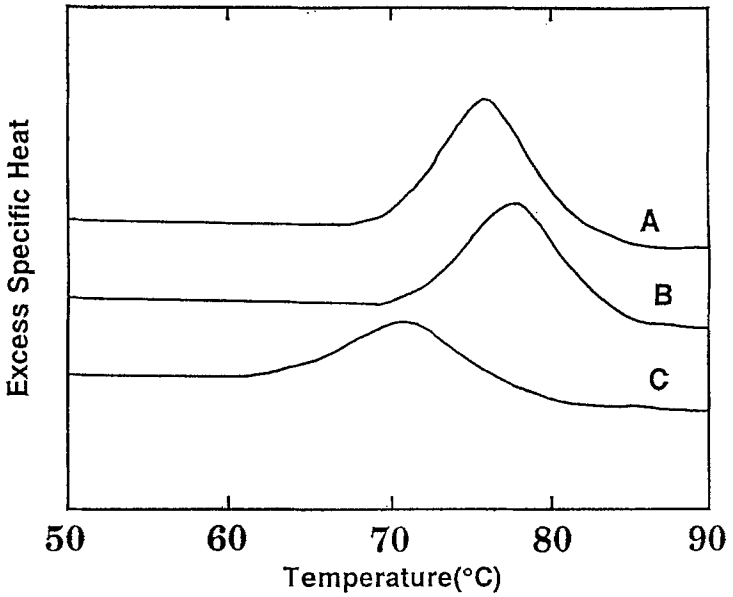

Figure 3. DSC data for native cytochrome b_{562}, G64A, and 22C/82C. These results were obtained in 10 mM phosphate buffer. The excess specific heat as a function of temperature was measured with a DASM-1 microcalorimeter. The temperature was increased at a rate of 0.5°C/min from 5 to 90°C. A: native cyt b_{562}, B: G64A, C: 22C/82C.

stabilities of these mutants with oxidized heme were examined and were compared with that of the wild-type protein with oxidized heme. Thermally induced denaturation of cyt-b_{562} was monitored by the UV-Vis (Soret band at 418 nm) and CD (at 222 nm) spectral changes, and the differential scanning microcalorimetry (DSC) measurement. Each can follow the dissociation of the heme moiety, the transition from α-helix to random coil and the thermal transition of heat capacity, respectively. The results of UV-Vis, CD, and DSC measurements indicate that the dissociation of the heme moiety initiates the denaturation of α-helices. It is clear from Figure 3 that the denaturation temperature of G64A mutant is almost 78°C and is 2° higher than that of wild-type, whereas that of A22C/G82C mutant is about 10° lower. It is suggested that the unexpected instability in A22C/G82C mutant is due to the weak binding of the heme moiety to the peptide chain which was induced by structural distortion of apo-mutant protein.

CONCLUSIONS

Electron transfer protein cyt-b_{562} was chosen as target materials for bioelectronics and was modified genetically and chemically in order to increase stability and orientational control in two-dimensional crystallization by means of LB technique. These modifications of the protein were almost successful and the purpose was partially accomplished. More efforts are thus needed to develop techniques for two-dimensional crystallization and the protein design for constructing the frontier of future electronic devices.

ACKNOWLEDGMENT

The authors would like to express their thanks to Prof. S. G. Sligar (University of Illinois) for providing us over-expressed *Escherichia coli* strain, and Drs. H.Uedaira and H.Morii (National Institute of Materials and Chemical Research) for carrying out the CD and DSC analyses.

REFERENCES

1. E. Itagaki and L. P. Hager, *J. Biol. Chem.*, **241**, 3687 (1966).
2. F. Lederer, A. Glatigny, P. H. Bethge, H. D. Bellany and F. S. Mathews, *J. Mol. Biol.*, **148**, 427 (1981).
3. F. S. Mathews, P. H. Bethge and E. W. Czerwinski, *J. Biol. Chem.*, **254**, 1699 (1979).
4. T. Furuno, H. Sasabe and K. M. Ulmer, *Thin Solid Films*, **180**, 23 (1989).
5. C. Mitchinson and J. A. Wells, *Biochemistry*, **28**, 4807 (1989).
6. L. J. Perry and R. Wetzel, *Science*, **226**, 555 (1984).
7. Y. Kobayashi, H. Sasabe and N. Saito, *J. Protein Chem.*, **12**, 121 (1993).
8. H. Nikkila, R. B. Gennis and S. G. Sligar, *Eur. J. Biochem.*, **202**, 309(1991).
9. F. W. J. Teale, *Biochem. Biophys. Acta*, **35**, 543 (1959).
10. E. Itagaki, G. Palmer and L. P. Hager, *J. Biol. Chem.*, **242**, 2272 (1967).