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Relationship between color development and protein conformation in the phycocyanin molecule

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Abstract

Phycocyanin with blue color, phycocyanobilin bound to protein, with the absorption maximum at 614 nm is quite unstable against urea-treatment because of the denaturation of the protein conformation. To prevent the bleaching of phycocyanin by urea, amino groups of lysine residues in the phycocyanin molecule were modified with DSP [dithiobis(succinimidyl propionate)] to cross-link amino groups in the protein molecule in order to maintain the high order structure of phycocyanin. The color development of DSP-modified phycocyanin became stable, but unmodified phycocyanin was markedly decreased to 20% of the original absorbance at 614 nm against urea-treatment. Splitting of the S–S linkage of DSP-modified phycocyanin molecule by 2-mercaptoethanol caused the reduction of color development of phycocyanin under urea-treatment. The color development of phycocyanin is closely associated with its high order structure of protein.

Keywords: Phycocyanin; Phycocyanobilin; Protein conformation; Dithiobis(succinimidyl propionate); Bio-conjugate

1. Introduction

Phycocyanin from *Spirulina platensis*, blue-green algae, is a protein composed of two kinds of subunits, α (20.5 kDa) and β (23.5 kDa), to form $\alpha_3\beta_3$ and nine phycocyanobilin moieties as a chromophore [1] (Fig. 1). The phycocyanin molecule has bright blue color with the absorption maximum at 614 nm. However, the color of phycocyanin is unstable to heat- or urea-treatment, because of the denaturation of the protein conformation. Although the detailed three-dimensional structure of phycocyanin was determined by X-ray crystallography [2,3], the relationship between the protein conformation and color development has not been satisfactorily clarified. The primary structures of α and β subunits of phycocyanin molecule were already determined in 1978

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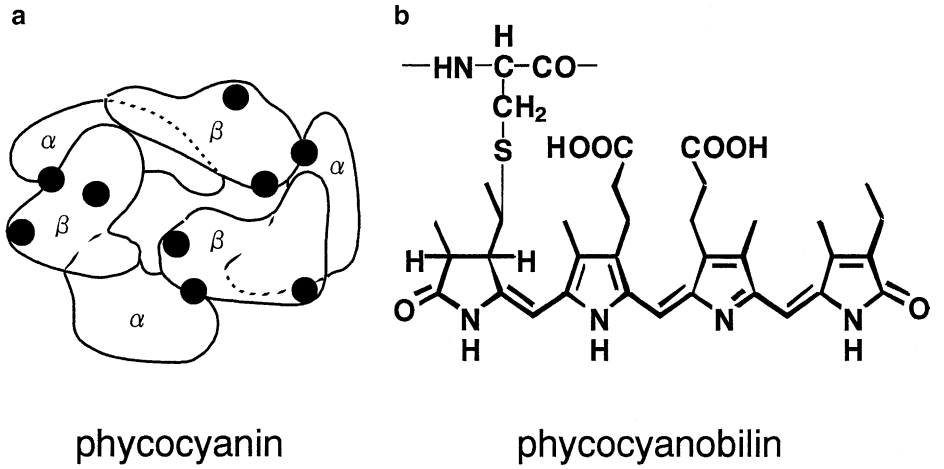


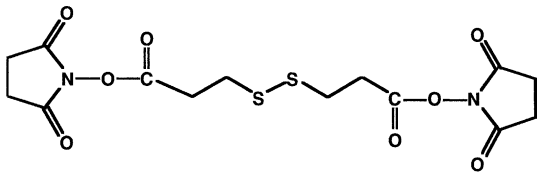
Fig. 1. The structures of phycocyanin (a) and phycocyanobilin (b). (a) Phycocyanin molecule composed of two kinds of protein (α and β subunits) to form $\alpha_3\beta_3$ and phycocyanobilin chromophore (●). (b) Chemical structure of phycocyanobilin, one on α-chain and two on β-chain.

by Freindenreich P. et al. [4,5]. Various kinds of proteins have been modified to stabilize the protein conformation using polyethylene glycol derivatives [6–8]. Moreover, the natural pigment, chlorophyll *a*, adsorbed on synthetic clay mineral, smectite, has shown a remarkable stability towards light-irradiation [9]. The present study deals with the relationship between the protein conformation and color development of phycocyanin composed of protein and chromophore of phycocyanobilin.

2. Experimental

Phycocyanin was isolated from *Spirulina platensis*, blue-green algae, obtained from Spirulina Bio-Lab. Co. Ltd., (Osaka, Japan) by the method of Kageyama et al. [10]. Dried powder (10 g) of *Spirulina platensis* was dissolved in 50 mM phosphate buffer, pH 7, (200 ml) and the sample solution was adjusted at isoelectric pH 4.0 of phycocyanin by adding 1.0 M citric acid. The precipitate obtained by centrifugation was dissolved in 50 mM phosphate buffer, pH 7, and the purified phycocyanin was obtained by filtration with DEAE resin, DEAE-TOYOPEARL 650 (TOSOH, Tokyo, Japan). Thus, purified powder of phycocyanin was obtained in a freeze-dried state. Subsequently, phycocyanobilin chromophore bound to phycobiliprotein was isolated from phycocyanin under reflux with methanol [11]. Dried phycocyanin powder (2.8 g) was refluxed in 200 ml of methanol with stirring at 65 °C for 16 h. Powder of phycocyanobilin was obtained by drying the solution under evaporation. Dithiobis(succinimidyl propionate), DSP (Lomant's reagent), was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA) [12].

D S P [dithiobis (succinimidyl propionate)]



molecular weight: 402.42 spacer arm 12 Å

3. Results and discussion

The absorption spectrum of phycocyanin (2×10^{-6} M) in water was shown in Fig. 2.

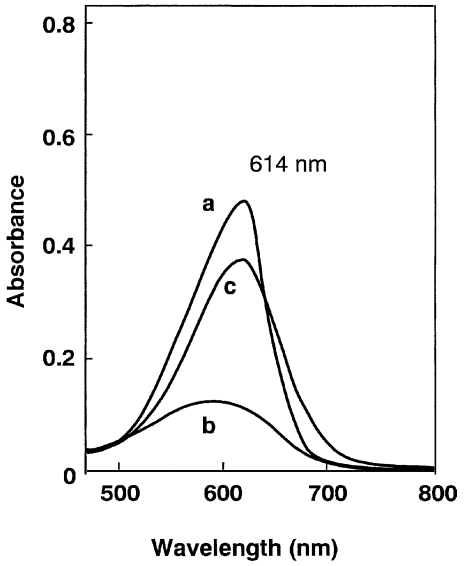


Fig. 2. Absorption spectra of phycocyanin (2×10^{-6} M). Curve a: phycocyanin in water, Curve b: phycocyanin after treatment with 4 M urea for 8 h. Curve c: phycocyanin after dialysis with water to remove urea.

Although native phycocyanin has absorption maxima at 614 nm (Curve a), the spectrum was markedly reduced (20%) by 4 M urea-treatment for 8 h (Curve b) due to the denaturation of the protein conformation. Moreover, phycocyanin once treated with urea was recovered to 78% of absorbance of native phycocyanin (Curve c) by dialysis with water to remove urea.

To prevent the decolorization of phycocyanin by denaturation of protein and clarify that the color development of the phycocyanin was closely related to the protein conformation, the amino groups in the phycocyanin molecule were cross-linked by dithiobis(succinimidyl propionate), DSP (Fig. 3). To 19 ml of phycocyanin (3.0 mg/ml) in 50 mM phosphate buffer saline (PBS) (pH 7.4) was added DSP (4.1, 8.2 and 16.4 mg) dissolved in 1.0 ml of dimethyl sulfoxide (DMSO). The sample mixture was stirred at 4 °C for 1 h to complete the reaction and diluted with 1.0 ml of 1.0 M Tris–HCl buffer (pH 7.5) to stop the reaction. The phycocyanin molecule has total 45 amino groups of lysine residues in the molecule. DSP was reacted with amino groups in the phycocyanin molecule at varying molar ratio of DSP/$-NH_2$ to form DSP-cross-linked phycocyanin (DSP–phycocyanin), which is shown in Fig. 3. The degree of modification of amino groups in the phycocyanin molecule was determined by using sodium 2,4,6-trinitrobenzenesulfonate (TNBS) [13]. Fig. 4 shows the degree of cross-linkage of amino groups in the phycocyanin molecule against molar ratio of DSP and amino groups (DSP/$-NH_2$). In 1.0 of molar-ratio of DSP against an amino group in the phycocyanin molecule (DSP/$-NH_2$), the degree of cross-linkage of amino groups in phycocyanin molecule reached at approximate 60% (Fig. 4). The absorption spectrum of phycocyanin was not transformed by DSP-cross-linkage. Moreover, the fluorescence of DSP–phycocyanin was ever different from that of non-cross-linked phycocyanin (data not shown).

Next series of experiments are concerned with the relation between protein conformation and color development of the phycocyanin molecule. Fig. 5-I shows the color development of phycocyanin after cross-linkage with DSP (Curve b) and with non-cross-linked phycocyanins (Curve a) in the presence of 4 M urea. The absorbance at 614 nm in Curve a was sharply decreased with time and reached 20% of the original color development at 8 h-incubation in 4 M urea. Once decreased absorbance in Curve a was increased at approximately 80% (Fig. 5-I, open square) of the original absorbance by dialysis of the sample with water. On the other hand, the absorbance at 614 nm of cross-linked phycocyanin (Curve b) was retained at 70% of the original color development in 4 M urea for 8 h and was increased at 100% (Fig. 5-I, open circle) by dialysis with water. The spectral change in Curve b is shown in Fig. 5-II, in which spectra a, b, and c show DSP–phycocyanin, after urea treatment and after dialysis with water, respectively. Therefore, color development of cross-linked phycocyanin is completely retained by dialysis.

Moreover, to clarify the relationship between color development and protein conformation of the phycocyanin molecules, S–S linkage in DSP-cross-linked phycocyanin was cleaved by adding 50 mM 2-mercaptoethanol (Fig. 3). Subsequently, once stabilized, color development to 70% (Fig. 5-I, Curve b) with cross-linked phycocyanin

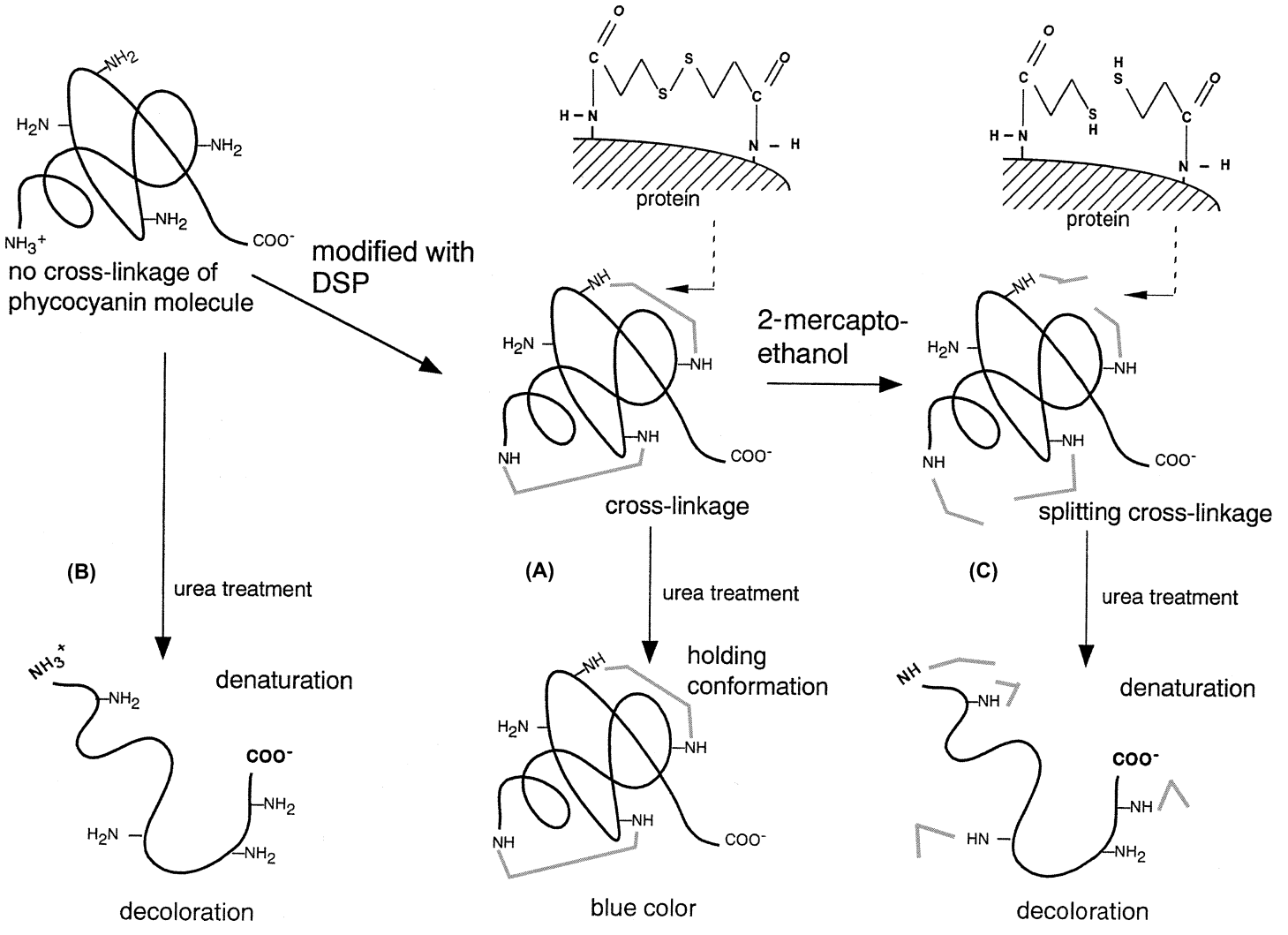


Fig. 3. The color development of phycocyanin with and without cross-linkage of amino groups in the phycocyanin molecule with DSP in 4 M urea. DSP-cross-linked phycocyanin retained the color development (blue color) even in urea (A). Color development of non-modified phycocyanin was lowered by denaturation with 4 M urea (B). Splitting of cross-linked phycocyanin led to decoloration by 4 M urea (C).

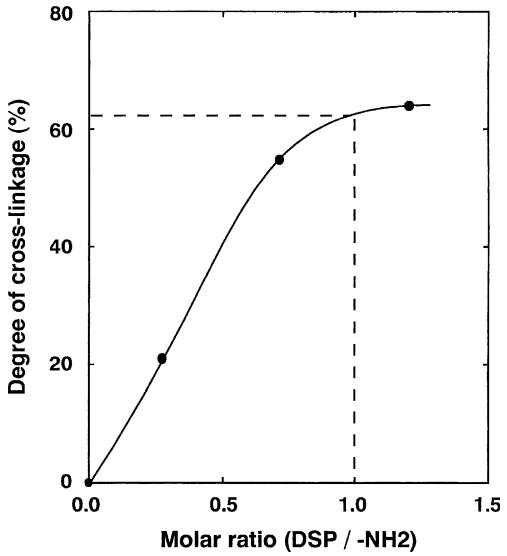


Fig. 4. Degree of cross-linkage of amino groups ($-NH_2$) in the phycocyanin molecule with DSP, against molar ratio of DSP/$-NH_2$ to form DSP-cross-linked phycocyanin.

at 8 h-incubation was reduced to 40% by splitting the cross-linkage with 2-mercaptoethanol (Fig. 5-I, open triangle). Through the data obtained from phycocyanin with blue color, it can be concluded that the high order structure of phycocyanin ($\alpha_3\beta_3$) is closely related to the color development of phycocyanobilin.

Anfinsen reported that the unique three-dimensional structure of ribonuclease was strongly related to the physiological function [14]. Moreover, many investigators reported the reversibility of denaturation and renaturation of protein conformations in relation to their functions [15,16]. Therefore, it may be concluded that the color development of phycocyanin molecule is maintained by the high order structure of protein molecule. A similar phenomenon has been observed for hemoglobin with red color composed of heme and globin; denaturation of globin protein may be related to discolorment of heme [17].

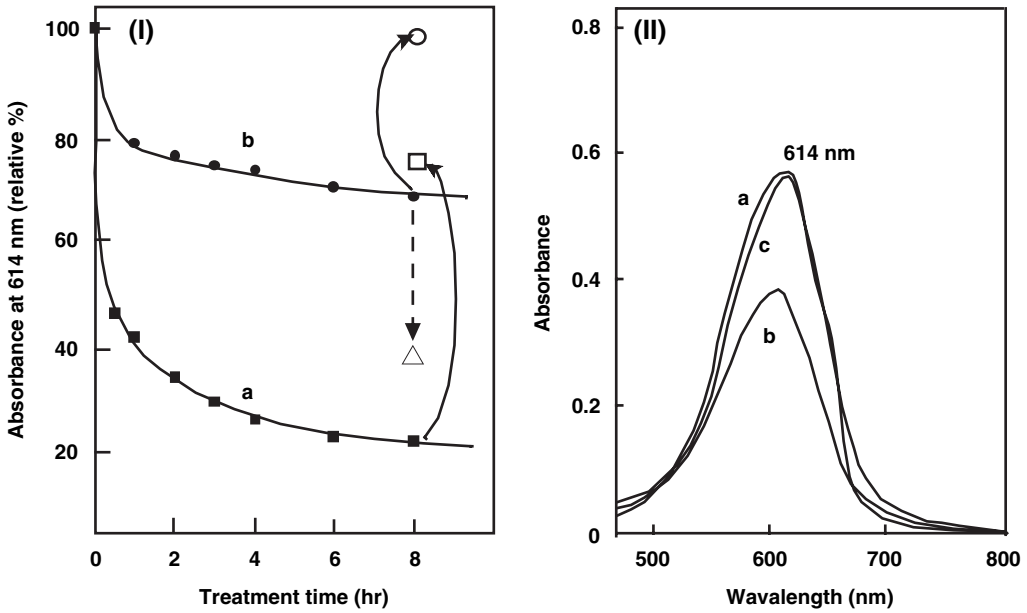


Fig. 5. (I) The absorbance decrease at 614 nm of non-cross-linked phycocyanin (Curve a) and of DSP-cross-linked phycocyanin (Curve b) with time in 4 M urea. Absorbance of non-cross-linked phycocyanin (open square, □) and DSP-cross-linked phycocyanin (open circle, ○) after dialysis with water after 8 h of urea-treatment. The absorbance of cross-linked phycocyanin at 614 nm was reduced to 40% from 70% by splitting cross-linkage (open triangle, △) with 2-mercaptoethanol in 4 M urea. (II) Spectra of DSP–phycocyanin (Curve a) and of DSP–phycocyanin with 4 M urea after 8 h (Curve b) and of DSP–phycocyanin after dialysis to remove urea (Curve c).

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