In vitro assembly of a β_2 cytochrome b_{559}-like complex from the chemically synthesised β-subunit encoded by the *Synechocystis* sp. 6803 *psbF* gene

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Abstract The α- and β-subunits of cytochrome b_{559} encoded by the *psbE* and *psbF* gene, respectively, are essential components of photosystem II. The exact structure of this cytochrome is not yet known. The β-subunit of the *Synechocystis* sp. 6803 cytochrome b_{559} complex was synthesised by means of solid-phase peptide synthesis. Under reducing conditions, two β-peptide molecules could be assembled specifically with one haem to form a β_2 cytochrome b_{559}-like complex. The spectral properties and the midpoint redox potential (48 ± 5 mV) of the in vitro assembled β_2 cytochrome are nearly identical to those of the low potential form of the native cytochrome b_{559}.

Key words: Assembly; Cytochrome b_{559}; Photosystem II

1. Introduction

Cytochrome b_{559} is one of the essential components of the photosystem II reaction centre. While it is established that the presence of cytochrome b_{559} is a prerequisite for the assembly of photosystem II [1–3], the function of this cytochrome is not yet clearly understood. A remarkable feature of the protein is that it can assume two different redox potentials, a low potential (20–80 mV) and an exceptionally high potential (330 and 400 mV) [4]. In contrast to other *b*-type cytochromes that participate in electron transfer reactions, no direct evidence is available to indicate that cytochrome b_{559} plays a functional role in the linear electron transfer processes within photosystem II [5]. Indirect evidence strongly indicates that cytochrome b_{559} may participate in side reactions involving the transfer of electrons, for example the protection of photosystem II against photodamage (reviewed in [5,6]).

Two short polypeptides, α and β, constitute cytochrome b_{559}. The genes encoding these peptides, *psbE* and *psbF*, respectively, are part of one operon in higher plants [7] and cyanobacteria [1], while in *Chlamydomonas reinhardtii* the genes occupy different locations on the chromosome and are therefore transcribed separately [8]. The amino acid sequences reveal that both polypeptides contain a single histidine, which is located at an identical position in the predicted membrane spanning α-helical domain [1,7]. Two histidine residues function as the axial ligands of the haem in cytochrome b_{559}, as shown by EPR and resonance Raman measurements [9]. Therefore it was suggested that cytochrome b_{559} is assembled from the α- and β-peptide linked in a parallel orientation by one haem [10]. However, based on the available data the existence of α_2- and β_2-cytochrome b_{559} cannot be excluded. Moreover, recent experiments of McNamara et al. [11] seem to favour the latter possibility.

In this work we have explored the feasibility of in vitro reconstitution of a homodimeric β_2 cytochrome b_{559} from the chemically synthesised β-peptide as encoded by the *Synechocystis* sp. 6803 *psbF* gene sequence.

2. Materials and methods

Solid-phase peptide synthesis in a Milligen Model 9050 peptide synthesiser began with Fmoc-Arg(Pbf)-PEG-PS preloaded resin (Perseptive). A four-fold excess of Fmoc L-amino acids was activated with HATU/DIEA. Two coupling cycles were used only for tryptophan with Fmoc-Trp(Boc). Each cycle was terminated by capping with 5% (v/v) acetic anhydride in DMF. After cleavage from the resin with 90/5/2/3 (v/v/v/w) TFA/thioanisole/anisole/DTT and precipitation with diethyl ether, the peptide was purified by preparative HPLC [12] using a gradient of 50–70% acetonitrile in water with 0.1% TFA over 30 min. After identification by mass spectrometry the fraction with the peptide was purified again with the settings of the first run, and then lyophilised.

For in vitro assembly of the cytochrome, 10 μl of a 500 mM Triton X-100 (BDH) solution, 15 μl distilled water, 10 μl of a solution containing 500 mM Bis-Tris propane-HCl (pH 7.0), 0.9 μl of a 10 mM solution of haem (bovine; Sigma) in 100% DMSO (Merck) and 10 μl of a solution of the cytochrome b_{559} β-peptide (10 mg/ml) in 25% LDS (Serva), were mixed and then 5 μl of a freshly prepared solution of sodium dithionite (100 mM; Sigma) was added. This mixture was further diluted in a buffer containing 50 mM Bis-Tris propane-HCl (pH 7.0), 1 mM Triton X-100 and 0.1 mM LDS which was made oxygen-free with argon.

Absorption spectra were measured on an Aminco DW2000 spectrophotometer. Redox titrations were performed at 20°C. Potentials were measured with a platinum electrode (Ingoldt Pt-4800 M5, with internal Ag/AgCl reference electrode), which was calibrated with quinhydrone (Sigma) and potassium ferri- and ferrocyanide. The following mediators (30 μM) were used: 1,4-naphthoquinone; duroquinone; 2-methyl-1,4-naphthoquinone; 2,3-dimethoxy-5-methyl-1,4-benzoquinone; 2-isopropoxy-1,4-naphthoquinone; and (15 μM) 2,3,5,6-tetramethyl-1,4-phenylenediamine; 1,2-naphthoquinone; phenazine ethosulfate; 2-hydroxy-1,4-naphthoquinone; anthraquinone-2-sulfonate. The mediators were purchased from either Aldrich or Sigma. Sodium dithionite and potassium ferricyanide were used as reductant and oxidant, respectively. The sample was kept free of oxygen by a stream of argon which was directed over the solution.

3. Results

The β-peptide of the cytochrome b_{559}, as encoded by the *psbF* gene of *Synechocystis* sp. 6803, was synthesised by automated solid-phase peptide synthesis. Electrospray mass spec-

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Abbreviations: LDS, lithium dodecylsulfate; OG, octylglucoside; SDS, sodium dodecylsulfate

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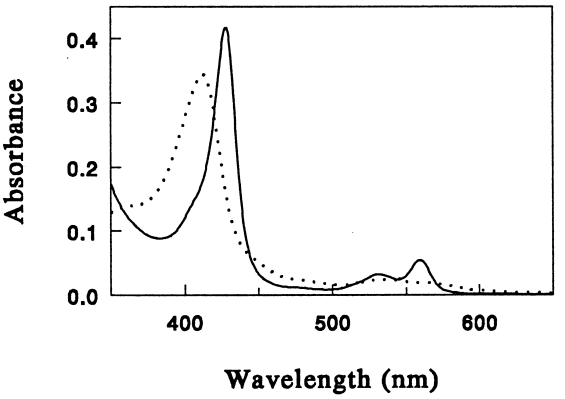

Fig. 1. Absorption spectrum of β_2 cytochrome b_{559} in reduced (solid line) and oxidised (dotted line) form. The oxidised spectrum was obtained by adding small amounts of potassium ferricyanide to the reduced protein. The sample was kept anaerobic with argon.

trometry yielded a mass of 4933 Da, in agreement with that of 4932 Da calculated from the amino acid sequence.

Our aim in this work was to probe whether it is possible to assemble a homodimeric *b*-type cytochrome from the chemically synthesised peptide. To this end the detergent-solubilised peptide was added to a solution containing detergent and haem, and the changes in the absorption spectrum (caused by the assembly) were monitored. The absorption spectrum was compared with that of 'free' haem in detergent and that of isolated native cytochrome b_{559} as reported in the literature [9,13]. A red shift in the position of the γ-band towards the maximum exhibited by the isolated native cytochrome b_{559} and a decrease of the width of this band were used as indications of improved assembly. An absorption band located at 600 nm (oxidised) or 580 nm (reduced) was considered an indicator of non-bound haem. Assembly trials were made with the following detergents: sodium deoxycholate, sodium dodecylsulfate (SDS), lithium dodecylsulfate (LDS), *N*-dodecyl-*N*,*N*-dimethyl ammonio-3-propane sulfonate, octylglucoside (OG), Triton X-100, LDAO and Lubrol PX, in the presence or absence of sodium dithionite. The choice of detergent and the redox state of the haem appeared to be critical for assembly. To our surprise, only the peptide solubilised in LDS or SDS together with reduced haem solubilised in OG or Triton X-100 yielded a protein with an absorption spectrum similar to that of isolated native cytochrome b_{559}.

The absorption spectrum of the assembled protein in the reduced and in the oxidised state is shown in Fig. 1. In Table 1 the spectral properties of the in vitro assembled cytochrome are compared with those published for the natural cytochrome

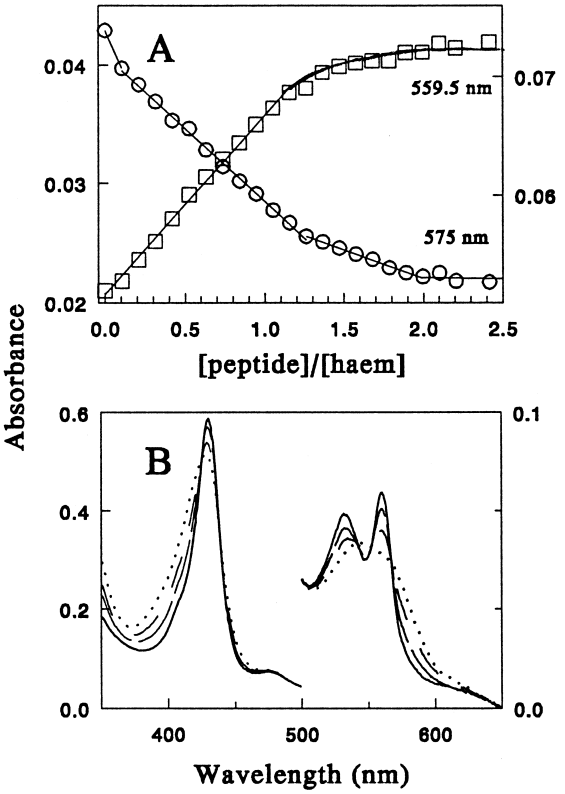

Fig. 2. Absorption of 4.2 μM ferro-haem in a solution containing 50 mM Bis-Tris propane (pH 7.0) and 20 mM Triton X-100 after the addition of small quantities of the chemically synthesised β-peptide solubilised in 25% LDS (10 μg/μl). A: Monitored at 559.5 nm (□) and 575 nm (○), the maximum of the α-band of the assembled protein and a wavelength were only 'free' haem absorbs, respectively. B: Spectrum after the addition of 0 (dotted), 3.4 (short dashes), 5.0 (long dashes) and 9.2 (solid) μM of the β-peptide (from 350 to 500 nm scaled left and from 500 to 650 nm scaled right). The sample was kept reduced with sodium dithionite (1 mM) and anaerobic with argon.

b_{559} in its isolated form. Besides a red shift of 1 nm of the γ-band, the only difference appears to be a higher relative intensity of the β-band in the spectrum of the reduced artificial cytochrome. The narrow width of the absorption bands indicates the haem is in a well-defined environment.

The assembled protein remains strongly associated while the haem is reduced. Neither storage for 2 days at room temperature in a reducing and oxygen-free environment nor the addition of 4 M of guanidine-HCl led to changes in the absorption spectrum. However, upon oxidation by potassium ferricyanide the γ-band shifted from 413 nm to 409 nm in

Table 1
Absorption maxima (in nm) and their relative intensities (in parentheses) of isolated native cytochrome b_{559} (in vivo data from [9]) and the in vitro assembled β_2 cytochrome b_{559}

	γ-band	β-band	α-band	fwhm γ
cyt b_{559}				
Ox	413 (0.82)	534 (0.068)	562 (0.057)	36
Red	427 (1.00)	530 (0.072)	559 (0.157)	25
β_2 cyt b_{559}				
Ox	413 (0.81)	534 (0.069)	560–570 (0.057)	41
Red	428 (1.00)	530 (0.104)	559.5 (0.157)	22

The spectra were normalised at the γ-band in the reduced spectrum. The full width at half maximum (fwhm, in nm) of the γ-band is listed in the last column.

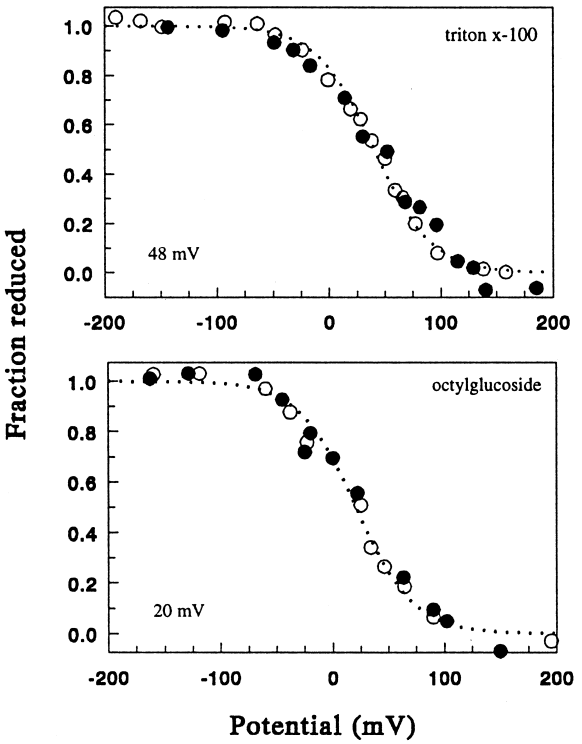

Fig. 3. Redox titration of the β_2 cytochrome b_{559} in OG and Triton X-100 micelles. The titrations were performed in reductive (●) and oxidative (○) sequence. The absorption difference between 559 and 570 nm was taken to represent the 'state' of reduction. The data were fitted with the Nernst equation ($n = 1$) and the scale of the ordinate normalised to the fraction of reduced β_2 cytochrome b_{559}.

several hours and an absorption band started to appear around 600 nm, when Triton X-100 was used for solubilisation of the haem. When OG was used for the solubilisation and assembly of the cytochrome these changes were immediate and the γ-band even shifted to a lower wavelength. Oxidation by oxygen led to a considerable absorption decrease, probably caused by self-catalysed breakdown of haem.

To establish the ratio protein/haem in the assembled complex, the association of peptide and haem was studied by titrating a solution containing reduced haem with small quantities of the chemically synthesised β-peptide and simultaneous recording of the resulting absorption spectra. The results of the experiment are shown in Fig. 2. Every addition of peptide caused an instantaneous change in absorption. When the number of peptide molecules per haem surpassed 2.0 there was no further change (see Fig. 2A). This indicates that two peptide molecules bind exactly one haem. Hence the protein can be referred to as a β_2 cytochrome b_{559}. The occurrence of clear isosbestic points in the absorption spectra (see Fig. 2B) implies that the association is highly specific.

On the basis of the previous experiment the extinction coefficient of β_2 cytochrome b_{559} could be calculated. The extinction coefficient of the α-band of the reduced protein was $\varepsilon_{559.5} = 17.4 \pm 0.7$ mM^{-1} cm^{-1}. This number is somewhat smaller than those reported in the literature for isolated native cytochrome b_{559}: $\varepsilon_{red-ox} = 17.5$ mM^{-1} cm^{-1} [4] and $\varepsilon_{559} = 21$ mM^{-1} cm^{-1} [14].

The redox potential was determined for protein that was assembled with haem solubilised in Triton X-100 as well as in OG. The results of the corresponding redox titrations are shown in Fig. 3. The midpoint potential of β_2 cytochrome b_{559} was 48 ± 5 mV in Triton X-100 and 20 ± 6 mV in OG. The absorption spectra related to the redox titrations showed isosbestic points at 420, 441, 520 and 570 nm down to about −50 mV. At lower potential the peaks became somewhat higher and broader. A similar absorption increase was also observed with isolated native cytochrome b_{559} upon reduction with sodium dithionite [13].

4. Discussion

This paper describes the successful chemical synthesis of the β-peptide of cyanobacterial cytochrome b_{559} and its assembly. We show that two of these chemically synthesised β-peptides associate in a specific manner with exactly one haem to form a β_2 cytochrome b_{559}. Surprisingly, assembly is only successful with ferro-haem. Except for the intensity difference of the β-band, the absorption spectrum of the reduced protein is identical to that of isolated native cytochrome b_{559}. However, upon oxidation the spectrum changes and acquires characteristics of the absorption spectrum of 'free' haem; a band at 600 nm appears and the γ-band shifts from 413 nm to a lower wavelength. This indicates that in the homodimeric β_2 cytochrome b_{559} the haem becomes more exposed to the environment upon oxidation. The extent of the absorption changes shows that the 'exposure' is more pronounced in OG micelles than it is in Triton X-100 micelles, which are much bigger. At the same time the midpoint potential of the protein is about 30 mV lower in OG than it is in Triton X-100 micelles. The decrease of the midpoint potential for a β_2 cytochrome b_{559} in which the haem is more exposed is in line with the observations of Matsuda and Butler [15] who transformed low-potential into high-potential cytochrome b_{559} by its reconstitution in liposomes, and with the model of Krishtalik et al. [16] for the influence of the haem environment on the redox potential. Preliminary experiments indicated that the β_2 cytochrome b_{559} could be introduced into single-walled liposomes formed from bacterial lipids (data not shown), but we were not yet able to test the effect of liposomes on the midpoint potential of the β_2 cytochrome b_{559}. In a synthetic cytochrome b with two bis-histidine-ligated haem groups [12] the midpoint potential showed values of −170 and −106 mV which are considerably lower than that of our β_2 cytochrome b_{559}. However, in that approach the hydrophobic helices of the mimicked protein had been converted to amphiphilic ones which assembled to a water-soluble four-helix bundle.

As mentioned already β_2 cytochrome b_{559} is more stable in its reduced than in its oxidised state. This property was also observed with isolated cytochrome b_{559} [14]. In contrast, the ferri form of the synthetic water-soluble cytochrome b referred to above had higher stability than the ferro form (Rau and Haehnel, unpublished).

To our knowledge this is one of the first examples of a membrane protein composed of chemically synthesised peptide. Our results indicate that in vitro assembly of homodimeric β_2 cytochrome b_{559} exhibiting spectral and redox properties nearly identical to those of the native low-potential cytochrome b_{559} is possible and support our future attempts aimed at testing the feasibility of assembling the homodimeric α_2 as well as the heterodimeric αβ complex and testing their properties.

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