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# Purification and properties of the intact P-700 and $F_X$-containing Photosystem I core protein

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**The intact Photosystem I core protein, containing the *psaA* and *psaB* polypeptides, and electron transfer components P-700 through $F_X$, was isolated from cyanobacterial and higher plant Photosystem I complexes with chaotropic agents followed by sucrose density ultracentrifugation. The concentrations of $NaClO_4$, NaSCN, NaI, NaBr or urea required for the functional removal of the 8.9 kDa, $F_A/F_B$ polypeptide was shown to be inversely related to the strength of the chaotrope. The Photosystem I core protein, which was purified to homogeniety, contains 4 mol of acid-labile sulfide and has the following properties: (i) the $F_X$-containing core consists of the 82 and 83 kDa reaction center polypeptides but is totally devoid of the low-molecular-mass polypeptides; (ii) methyl viologen and other bipyridilium dyes have the ability to accept electrons directly from $F_X$; (iii) the difference spectrum of $F_X$ from 400 to 900 nm is characteristic of an iron-sulfur cluster; (iv) the midpoint potential of $F_X$, determined optically at room temperature, is 60 mV more positive than in the control; (v) there is indication by ESR spectroscopy of low-temperature heterogeniety within $F_X$; and (vi) the heterogeneity is seen by optical spectroscopy as inefficiency in low-temperature electron flow to $F_X$. The constraints imposed by the amount of non-heme iron and labile sulfide in the Photosystem I core protein, the cysteine content of the *psaA* and *psaB* polypeptides, and the stoichiometry of high-molecular-mass polypeptides, cause us to re-examine the possibility that $F_X$ is a [4Fe-4S] rather than a [2Fe-2S] cluster ligated by homologous cysteine residues on the *psaA* and *psaB* heterodimer.**

## Introduction

The Photosystem I reaction center of green plants and cyanobacteria is a multiprotein complex that functions as a light-driven plastocyanin-ferredoxin oxidoreductase. The electron-transfer components include a chlorophyll primary electron donor, P-700, a chlorophyll primary electron acceptor $A_0$, a quinone intermediate electron acceptor, $A_1$, and three iron-sulfur centers, $F_A$, $F_B$ and $F_X$ (see Refs. 1–3 for review). According to current understanding, a singlet exciton migrates from an excited antenna chlorophyll to the reaction-center trap, bringing about charge separation between P-700 and $A_0$. The electron is then passed through the intermediate acceptors $A_1$ and $F_X$ to the terminal electron acceptors $F_A/F_B$. In the absence of ferredoxin and plastocyanin, the charge separation is unstable, and $[F_A/F_B]^-$ backreacts with $P\text{-}700^+$ within 30 ms.

The Photosystem I complex, which contains P-700 and the electron acceptors $A_0$ through $F_A/F_B$, can be isolated from plant and cyanobacterial membranes with the use of non-ionic detergents such as digitonin, octyl glucoside, and Triton X-100. The polypeptide complement of this complex falls into two general molecular mass ranges as determined by SDS-PAGE: in the high range, two related polypeptides are observed at 55–65 kDa (which correspond to the 82 and 83 kDa reaction center polypeptides), and in the low range, two to five polypeptides are observed from 8 to 21 kDa, depending on the species and the degree of purification of the

Abbreviations: PS I, Photosystem I; Chl, chlorophyll; LDS, lithium dodecyl sulfate; SDS, sodium dodecyl sulfate; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morphineethanesulfonic acid; DCPIP, 2,6-dichlorophenylindophenol; PMS, phenazine methosulfate; APS, ammonium persulfate; PAGE, polyacrylamide gel electrophoresis; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

Definitions: Photosystem I complex: multiprotein reaction center isolated from thylakoid membranes with Triton X-100, containing P-700 and electron acceptors, $A_0$, $A_1$, $F_X$, $F_B$ and $F_A$.
Photosystem I core protein: reaction center heterodimer of the *psaA* and *psaB* protein isolated from the Photosystem I complex with chaotropic agents, containing P-700 and electron acceptors $A_0$, $A_1$ and $F_X$.

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particle (higher plants also contain peripheral chlorophyll *a/b* light harvesting proteins [4]). The polypeptide location of the electron transport components is now known: $A_1$ [5–7] and $F_X$ [8–11] are associated with the P-700- and $A_0$-containing [12] reaction center polypeptides, and $F_A$ and $F_B$ are lcoated on a peripheral 8.9 kDa polypeptide [13–19].

The Photosystem I complex can be stripped of the low-molecular-mass proteins, including the $F_A/F_B$ polypeptide, by a brief exposure to 1% LDS [8–10]. Under these conditions, transient charge separation and recombination occurs between P-700 and $F_X$ without the need for prior reduction of $F_A$ and $F_B$. The optical absorption, electron spin resonance, and biochemical data indicate that $F_X$ is an iron-sulfur cluster closely associated with the reaction-center polypeptides. Unfortunately, LDS has a differential effect on the Photosystem I complex and produces a mixture of products: 20% of the reaction centers remain in the control state [P-700...$A_0A_1F_XF_BF_A$], 60% contain the desired core protein [P-700...$A_0A_1F_X$], and 20% are degraded to the CP1 state [P-700...$A_0$]. An equally significant problem is that the LDS-treated Photosystem I complex becomes unstable in the presence of non-ionic (as well as ionic) detergents, rendering further purification and handling difficult. The need for a homogeneous, intact Photosystem I core protein prompted us to seek an alternate method, which would totally deplete the reaction center of the $F_A/F_B$ polypeptide, but which would completely retain $F_X$.

We recently showed that 6.8 M urea is highly effective in removing the $F_A/F_B$ polypeptide from a cyanobacterial Photosystem I complex [11]. The purified Photosystem I core protein has no measurable ESR signals from chemically or photochemically reduced $F_A$ or $F_B$, and shows one optical kinetic phase, the $P\text{-}700^+$ $F_X^-$ backreaction, at room temperature. The purified core protein contains 4.6 mol of acid-labile sulfur per mole of P-700 and lacks the low-molecular-mass proteins, including the 8.9 kDa polypeptide associated with $F_A$ and $F_B$.

In this paper, we report that the action of urea is not unique: all of the low-molecular-mass polypeptides can be removed from higher plant and cyanobacterial reaction centers using a wide variety of chaotropic agents, including urea, NaBr, NaI, NaSCN and $NaClO_4$. We report the detailed properties of $F_X$ in a Photosystem I core protein isolated by treatment with chaotropes followed by ultracentrifugation in a sucrose gradient.

## Materials and Methods

The cyanobacterium *Synechococcus* PCC 6301 (*Anacystis nidulans*) was grown axenically in Krantz and Myers medium C [20] at 38 °C under continuous fluorescent (cool white) illumination at 60 $\mu E/m^2$ per s. Cultures were aerated continuously with 1% $CO_2$ in air, and harvested in late log growth at a density corresponding to 15 $\mu$g Chl/ml. Cells from 1–2 l of culture were pelleted by centrifugation and resuspended in 0.2–0.3 mg Chl/ml in medium consisting of 0.05 M Tris-HCl (pH 7.5) plus 20 mM $Na_2EDTA$. The concentrated cells were disrupted by passage at approx. 2 drops/s through a French presure cell at 110 MPa. Unbroken cells were removed from the eluate by centrifugation ($1000 \times g$ for 3 min). The supernatant was again centrifuged ($45\,000 \times g$ for 1 h) to pellet most of the membrane fraction. The pellet was resuspended in the same Tris-EDTA buffer to a concentration of approx. 1.5 mg Chl/ml. Membrane preparation steps were all performed at room temperature until immediately after cell breakage; subsequent procedures were at 4 °C unless otherwise indicated. The membrane preparation was frozen until ready for use, and could be maintained at −20 °C for several months with no appreciable loss in ability to extract active Photosystem I preparations. Photosynthetically active membranes of the thermophylic species of *Synechococcus* sp. (grown at 55 °C) were supplied by Dr. Jerry Brand (University of Texas, Austin).

The thawed membranes were pelleted by centrifugation ($45\,000 \times g$ for 1 h). The pellet was resuspended to 100 $\mu$g Chl/ml in 0.7 mM EDTA and 50 mM Tris (pH 7.4) for 20 min at 25 °C. After centrifugation ($25\,000 \times g$ for 1 h) the pellet was resuspended to 100 $\mu$g Chl/min in 50 mM Tris (pH 7.8) containing 0.01 m KCl and sonicated three times for 10 s intervals. After recentrifugation ($25\,000 \times g$ for 1 h), the pellet was resuspended to 100 $\mu$g Chl/ml in 50 mM Tris (pH 8.3) plus 0.2 M KCl and 1% Triton X-100. The suspension was incubated for 12 h and centrifuged at $27\,000 \times g$ for 30 min. The supernatant was concentrated to 30 ml over a YM-100 ultrafiltration membrane (Amicon) and centrifuged for 18 h at $113\,000 \times g$ (SW-27 rotor) in a 0.1–1 M sucrose gradient containing 50 mM Tris (pH 8.3) and 0.1% Triton X-100. The lower green band was isolated, dialyzed overnight against 50 mM Tris (pH 8.3), concentrated over a YM-100 membrane and recentrifuged on a 0.1–1 M sucrose gradient in the absence of Triton X-100. The lower green band was dialyzed overnight against 50 mM Tris (pH 8.3) concentrated to 1 mg Chl/ml and stored at −80 °C in 20% glycerol. For studies involving the effect of various chaotropes, the sample was incubated with the appropriate concentration of chaotrope (see Table I) at 250 $\mu$g Chl/ml. For studies of $F_X$ and the polypeptide composition of the reaction center proteins, the sample was treated at 250 $\mu$g Chl/ml for 10 min with 2 M NaI. The treated sample was dialyzed overnight in 50 mM Tris (pH 8.3), concentrated over a YM-100 membrane and centrifuged for 18 h at $113\,000 \times g$ (SW-27 rotor) in a 0.1–1 M sucrose gradient containing 50 mM Tris (pH 8.3) plus

0.1% Triton X-100. The lower green band, which contains the reaction center in the state [P-700...$A_0A_1F_X$], was removed, dialyzed against 50 mM Tris (pH 8.3) and stored at −80 °C in 20% glycerol.

Flash-induced absorption transients were determined at 698 and 820 nm [8]. The data were captured and averaged on a Nicolet 4094A Digital Oscilloscope and transferred to a Macintosh Plus computer for display, manipulation and storage. The sample was repetitively flashed with a Phase-R DL-1200 flashlamp-pumped dye laser (50 mJ pulse energy for 400 ns FWHM at 660 nm) or a PTI-2300 nitrogen laser (1.4 mJ pulse energy for 600 ps FWHM at 337 nm). The optical measurements were performed in a themostatically controlled (20 °C) 1.0 cm pathlength cuvette containing 5 μg Chl/ml, 0.033 mM DCPIP or PMS, and 1.7 mM ascorbate in 50 mM Tris (pH 8.3). The fast and slow phases of the absorption transient were evaluated by extrapolating the log plot of the slow phase to time zero and 'peeling' the composite curve to extract details of the fast phase. Redox titrations were performed as described in Ref. 10.

Low-temperature optical studies were performed in a 1 cm pathlength polystyrene cuvette containing 5 μg Chl/ml, 0.033 mM PMS and 1.7 mM ascorbate in 60% glycerol and 0.10 M Tris (pH 8.3). The cuvette was initially placed in an optically transparent Dewar 1 cm above the liquid nitrogen surface and placed in darkness. Few, if any, cracks developed in the frozen glass when the cooling was performed slowly. After 10 min of precooling, excess liquid nitrogen was added until the cuvette was completely submerged. After an additional 10 min, the excess liquid nitrogen was removed and the Dewar was placed in the spectrometer for optical measurements. A chromel/alumel thermocouple (Omega Engineering) was used to monitor the temperature.

ESR studies were performed on a Varian E-109 spectrometer equipped with an Air Products LTD liquid helium transfer cryostat. The spectrometer was interfaced to a Macintosh Plus computer via a Keithley digital voltmeter (Model 195A) and an IEEE-488 bus controller (I/O Tech Mac 488A) for signal averaging and baseline subtraction. The software was written in FORTH by Mr. Martin Corera. Sample temperatures were monitored with a thermistor situated directly below the sample tube. Light-minus-dark difference spectra were obtained by illuminating the sample with a 150 W Xenon lamp.

P-700 concentration was determined photochemically after a saturating flash. Chl was determined spectrophotometrically after acetone extraction. Iron was determined using inductively coupled plasma spectroscopy and acid-labile sulfide data was from Ref. 11.

Electrophoresis (PAGE) was performed in a 8 cm × 7 cm × 1.5 mm slab gel (Hoefer Model SE-200) containing a linear 10–15% polyacrylamide gradient (bis : acrylamide of 1 : 20). The control and chaotrope-treated Photosystem I particles (1 μg/ml protein) were incubated in 0.0625 M Tris (pH 6.8), 2% SDS, 10% glycerol and 5% β-mercaptoethanol for 24 h at 30 °C. The samples were applied to the stacking gel of 6% polyacrylamide containing 50 mM Tris-$H_2SO_4$ (pH 6.1), 0.1% SDS, 1 mg/ml APS and 1 μl/ml TEMED at a protein concentration of 20 μg/well. Electrophoresis was carried out at 20 °C at a constant current of 12 mA for 2.5 h. Gels were stained with Coomassie brilliant blue and scanned with an LKB laser densitometer. Molecular masses were calculated on the basis of the $R_f$ values of soluble proteins from 6.21 to 66.0 kDa (Sigma Nos. MW-SDS-7, MW-SDS-70 and MS-SDS-200) treated at 37 °C for 2 h and run in alternate wells. Cross-linking studies were performed by incubation of the Photosystem I particles with 2% glutaraldehyde for 10 min at 20 °C. Prior to cross-linking, the particles were treated at 62 μg for 10 h with pH 8.0 phosphate buffer containing 1% digitonin at 20 °C. Cross-linking was terminated by the addition of 1.0 M glycine, 0.5 M MOPS, 0.5 M Mes (pH 7.0). The solution was dialyzed in 50 mM Tris (pH 8.3) and concentrated to 1 mg Chl/ml over a YM-10 membrane. Molecular masses were determined by SDS-PAGE using a linear gradient of 3–10% polyacrylamide with a 3% stacking gel.

## Results

*Effect of chaotropes on the Photosystem I complex*

The flash-induced absorption transient in a Photosystem I complex from spinach or *Synechococcus* results from charge separation and recombination between P-700$^+$ and the terminal iron-sulfur clusters [$F_A/F_B$]$^-$. If the *Synechococcus* Photosystem I complex is treated with 6.8 M urea, the 30 ms backreaction is progressively replaced by a faster, monophasic absorption transient with a half-time of approx. 1.2 ms [11]. We have shown by spectroscopic and biochemical methods that the 1.2 ms half-time represents the P-700$^+$ $F_X^-$ backreaction in a reaction center deficient in the $F_A/F_B$ polypeptide [8–10].

Because urea reduces the ability of water to form hydrogen bonds, we tested several other chaotropes for their effectiveness in removing the $F_A/F_B$ polypeptide. Fig. 1 shows the flash-induced absorption transient at 698 nm at several time intervals after treatment of a *Synechococcus* Photosystem I complex with 2 M NaI. After 30 min of incubation, the 30 ms, P-700$^+$ [$F_A/F_B$]$^-$ backreaction is more than 90% replaced by the 1.2 ms, P-700$^+$ $F_X^-$ absorption transient with no apparent loss in signal amplitude. In Table I, the minimum concentration of chaotrope required to replace the P-700$^+$ [$F_A/F_B$]$^-$ transient with the P-700$^+$ $F_X^-$ transient is listed for three different Photosystem I preparations; spinach, *Synechococcus* (mesophilic species grown at

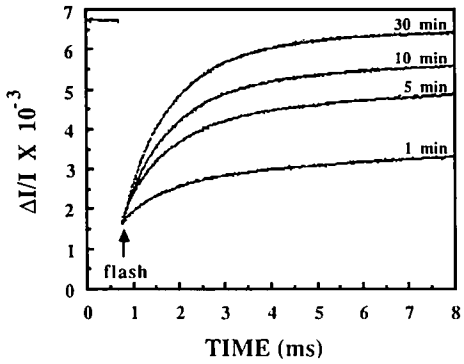


Fig. 1. Time-course of the 698 nm absorption transient at 298 K in a Photosystem I complex during treatment with 2 M NaI. The reaction mixture contained 5 μg/ml chlorophyll, 0.033 mM PMS and 1.7 mM sodium ascorbate. Excitation was provided by an 800 ps nitrogen laser. After 30 min of incubation, the 1.2 ms phase constitutes more than 90% of the total signal, indicating the retention of iron-sulfur center $F_X$, but the absence of iron-sulfur centers, $F_A$ and $F_B$.

38°C), and *Synechococcus* (thermophilic species grown at 55°C). The data indicate that the concentration required for the functional removal of $F_A$ and $F_B$ is inversely related to the strength of the chaotrope. Also of interest is the lower resistance of spinach, and the increased resistance of the thermophilic species of *Synechococcus* to chaotropic agents. In accord with these trends, we have found that $F_X$ is more labile in spinach than in *Synechococcus* and that complete removal of $F_A$ and $F_B$ is difficult to achieve in the thermophile. The mesophylic species of *Synechococcus* responds to chaotropes in a manner which allows the complete removal of $F_A$ and $F_B$ without any degradation of $F_X$.

The $F_X$-containing reaction center remains relatively stable in the continued presence of the chaotrope and deteriorates only slowly over the course of several hours. However, the reaction center is unstable in the presence of chaotropes plus detergents such as dodecyl maltoside, octyl glucoside or Triton X-100. If the chaotrope treatment is carried out in the presence of 0.1–1% Triton X-100, the 1.2 ms P-700$^+$ $F_X^-$ absorption transient does not develop fully, and the 30 ms P-700$^+$ $[F_A/F_B]^-$ absorption transient becomes gradually replaced with a 3 μs absorption transient. At 77 K, the

TABLE I

Minimal chaotrope concentration ($M$) required for functional removal of the terminal electron acceptors $F_A$ and $F_B$ from the Photosystem I reaction center

| | *Synechococcus* (mesophilic) | *Synechococcus* (thermophilic) | Spinach |
|---|---|---|---|
| Urea | 6.8 | 6.8 | 5 |
| NaBr | 6 | 6 | 4.7 |
| NaI | 2 | 4 | 2 |
| NaSCN | 2 | 3.5 | 1.5 |
| $NaClO_4$ | 2 | 4 | 1.3 |

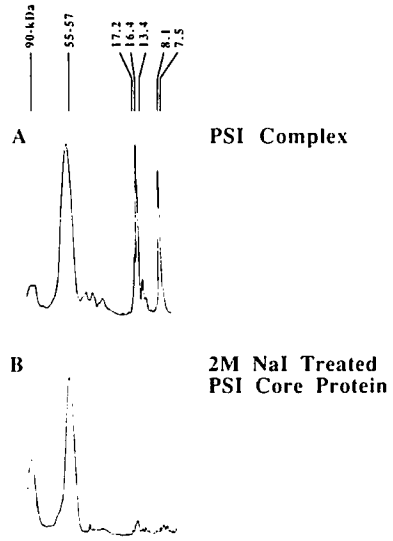


Fig. 2. Polypeptide composition of the Photosystem I complex and the Photosystem I core protein. (A) Laser-densitometric tracing of the Photosystem I complex after SDS-PAGE. (B) Laser-densitometric tracing of the Photosystem I core protein after SDS-PAGE following 30 min treatment with 2 M NaI and ultracentrifugation on a 0.1–1 M sucrose gradient. SDS-PAGE was performed in a 7.5–15% polyacrylamide gradient.

kinetics slow to 800 μs, indicating destruction of the iron-sulfur cluster and recombination of P-700$^+$ $A_O^-$ through the triplet state of P-700 [21]. The $F_X$-containing reaction center, however, is stable in the presence of chaotrope if the Triton X-100 concentration is lowered to less than 0.01%. This makes it possible to remove the chaotrope by gel filtration or overnight dialysis without significant degradation of the iron-sulfur cluster. Thereafter, the $F_X$-containing reaction center is stable in 0.01–0.1% Triton X-100 for at least 48 h at 4°C.

The $F_X$-containing reaction center can be purified to homogeneity by ultracentrifuging the chaotrope-treated and dialyzed Photosystem I complex in a 0.1% Triton-containing, 0.1 M sucrose gradient. After 18 h at 48000 × $g$, the complex separates into a colorless upper band, which contains the low-molecular-mass polypeptides (see below), and a lower green band, which contains the reaction-center polypeptides. Since the lower band has suffered little or no degradation of the P-700$^+$ $F_X^-$ reaction, and since it totally lacks $F_A$ and $F_B$ (see below), we have designated it the Photosystem I core protein [11].

*Components of the Photosystem I core protein*

The polypeptide composition of the Photosystem I complex and the Photosystem I core protein isolated using 2 M NaI are shown in Fig. 2. Under the specified gel conditions, the *Synechococcus* Photosystem I complex (Fig. 2a) contains a broad band at approx. 55–57 kDa corresponding to the monomeric reaction center polypeptides, and a minor chlorophyll-containing band at 90 kDa. Two prominent low molecular-mass poly-

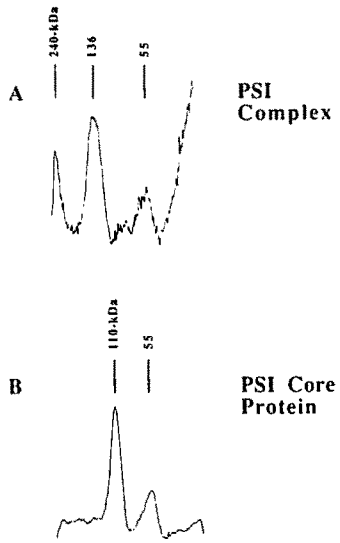


Fig. 3. Polypeptide composition of the cross-linked Photosystem I complex and Photosystem I core protein. (A) Laser-densitometric tracing of the cross-linked Photosystem I complex after SDS-PAGE. (B) Laser-densitometric tracing of the cross-linked Photosystem I core protein after SDS-PAGE. Both samples were incubated in treatment buffer containing 2% SDS and 5% β-mercaptoethanol for 24 h at 35°C.

peptides at 16.4 and 8.1 kDa are present as well as three low-abundance polypeptides at 17.2, 13.4 and 7.5 kDa (the latter is visible as a shoulder on the 8.1 kDa polypeptide and becomes prominent in partially depleted complexes). The persistence of the latter polypeptides in all preparations isolated to date indicates that the *Synechococcus* Photosystem I complex may contain as many as five low-molecular-mass polypeptides and that the 17.2, 13.4 and 7.5 kDa polypeptides have become partially depleted during the course of purification. In the chaotrope-treated and ultracentrifuged *Synechococcus* Photosystem I core protein (Fig. 2b), the 55–57 kDa band is present with the same intensity as in the Photosystem I complex, but the low-molecular-mass bands are totally missing. The 16.4 and 8.1 kDa polypeptides can be recovered from the YM-100 filtrate (see Materials and Methods) and purified to homogeniety by gel-filtration chromatography. However, the iron-sulfur clusters in the 8.9 kDa, $F_A/F_B$ polypeptide are unstable in the presence of chaotropes, and the protein is isolatable only with the chromophore in the sulfur-zero state (see Ref. 22).

The polypeptide compositions of the Photosystem I complex and the Photosystem I core protein after cross-linking with 2% glutaraldehyde are shown in Fig. 3. Under denaturing conditions, the Photosystem I complex (Fig. 3a) separates into three bands: a major band at 136 kDa, a minor band at 55 kDa and a band of variable intensity at approx. 240 kDa, all of which are devoid of chlorophyll. Since the latter band diminishes further on increasing incubation time with digitonin, and since even larger multiples of the 136 kDa band are observed in the absence of digitonin pretreatment, we suspect that aggregate formation is responsible for the high-molecular-mass band. After treatment with digitonin and cross-linking with glutaraldehyde, the Photosystem I core protein (Fig. 3b) resolves into two bands: a major band at 110 kDa and a minor band at 55 kDa, both of which are devoid of chlorophyll. The most significant feature is that the cross-linked Photosystem I complex is 26 kDa larger than the Photosystem I core protein, a difference which can be attributed to the chaotrope-induced loss of the 8.1 kDa and the 16.4 kDa polypeptides.

Accompanying the loss of these low-molecular-mass polypeptides is the loss of 8 mol each of non-heme iron and acid-labile sulfide. This results in a core protein that retains 4.6 mol of acid-labile sulfide and approx. 6 mol of total iron per mole of P-700. The iron content is somewhat variable and the excess over 4 mol may be derived from the denatured $F_A/F_B$ clusters. The chlorophyll-to-P-700 ratio is nearly unchanged from 108 Chl : P-700 in the Photosystem I complex to 110 Chl : P-700 in the Photosystem I core protein.

*Optical studies of the Photosystem I core protein*

The purification of an $F_X$-containing reaction center allowed us to compare directly the ability of $F_X^-$ and $[F_A/F_B]^-$ to donate electrons to artificial electron acceptors. Fig. 4 shows the extent of quenching of the $P\text{-}700^+$ $[F_A/F_B]^-$ and $P\text{-}700^+$ $F_X^-$ backreactions by methyl viologen for the Photosystem I complex and the Photosystem I core protein, respectively. $\Delta I/I$ represents the amount of long-lived $P\text{-}700^+$ and indicates the extent of donation from either $F_X^-$ or $[F_A/F_B]^-$ to the artificial electron acceptor. In the Photosystem I complex, the 30 ms back-reaction between $P\text{-}700^+$ and $F_A^-$ is almost totally suppressed at a methyl viologen concentration of 20 μM (Fig. 4a). However, in the Photosystem I core protein lacking $F_A$ and $F_B$, suppression of the 1.2 ms recombination between $P\text{-}700^+$ and $F_X^-$ requires a methyl viologen concentration of 2 mM (Fig. 4b). CP1 particles, which lack $F_A$, $F_B$ and $F_X$, require a methyl viologen concentration greatly in excess of 10 mM to suppress the recombination between $A_0^-$ and $P\text{-}700^+$ (data not shown). The concentration dependence is therefore governed by simple mass action and may be useful as a diagnostic tool in distinguishing between the various electron acceptors of Photosystem I.

Since methyl viologen can interact with the acceptors directly, it was possible to obtain a complete point-by-point difference spectrum of the acceptor responsible for the 1.2 ms backreaction: this should confirm that $F_X$ is an iron-sulfur cluster as indicated earlier by the ESR spectrum [9,10] and partial optical-difference spectrum [8]. The experimental protocol involved flash photolysis of a preparation containing (i) only ascorbate and

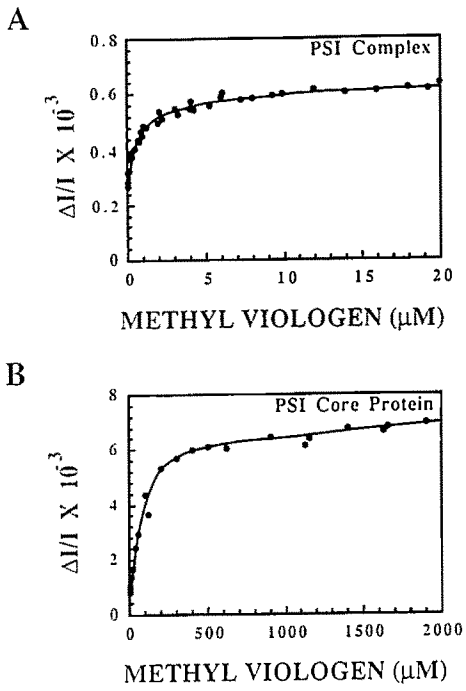


Fig. 4. Donation to methyl viologen by $F_X^-$ and $[F_A/F_B]^-$ in a Photosystem I complex and Photosystem I core protein. (A) Amplitude of the long-lived, 820 nm absorption component as a function of methyl viologen concentration in the Photosystem I complex. To minimize excitation of the antenna chlorophyll, 820 nm was chosen as the measuring wavelength and the concentration of DCPIP was reduced to 0.0033 mM. (B) Amplitude of the long-lived 698 nm absorption component as a function of methyl viologen concentration in the Photosystem I core protein. The measurements were performed at 5 μg/ml chlorophyll.

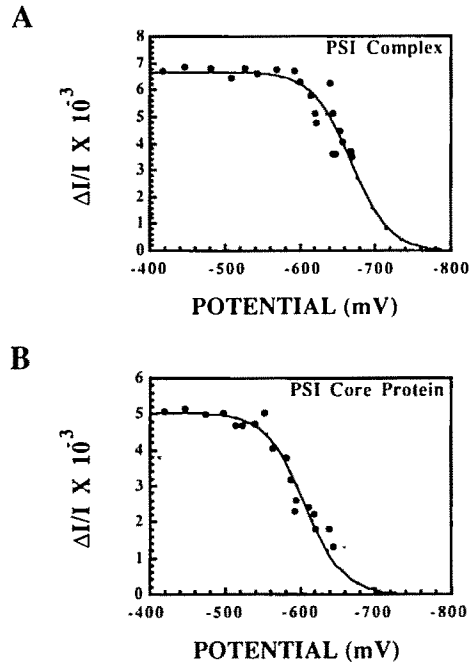


Fig. 6. Redox titration of $F_X$ in the Photosystem I complex and Photosystem I core protein. (A) Amplitude of the 698 nm absorption change (250 μs half-time) as a function of redox potential in the Photosystem I complex. The solid line represents a theoretical redox curve for a one-electron reduction with a midpoint potential of −670 mV. (B) Amplitude of the 698 nm absorption change (1.2 ms half-time) as a function of redox potential in the Photosystem I core protein. The solid line represents a theoretical redox curve for a one-electron reduction with a midpoint potential of −610 mV. The redox titrations were performed at 5 μg/ml and at 298 K.

DCPIP as electron donors and (ii) the same sample after addition of 2 mM methyl viologen. The difference in the absorption change between conditions (i) and (ii) corresponds to the differential absorption of the electron acceptor participating in the 1.2 ms backreaction with $P\text{-}700^+$. The spectrum (Fig. 5) shows the oxidizing species, P-700 (open diamonds), with two negative peaks at 435 and 698 nm, which is consistent with its identity as a chlorophyll. The reduced species (solid diamonds) shows a broad spectral band from 400 to 500 nm centered at 435 nm and a pronounced maximum near 430 nm, but lacking any significant bands beyond 480 nm. This difference spectrum is characteristic of an iron-sulfur cluster and is not compatible with either a quinone or a chlorophyll.

The half-time for the recombination between $P\text{-}700^+$ and $F_X^-$ in the Photosystem I core protein is 1.2 ms; however, the half-time for the recombination between

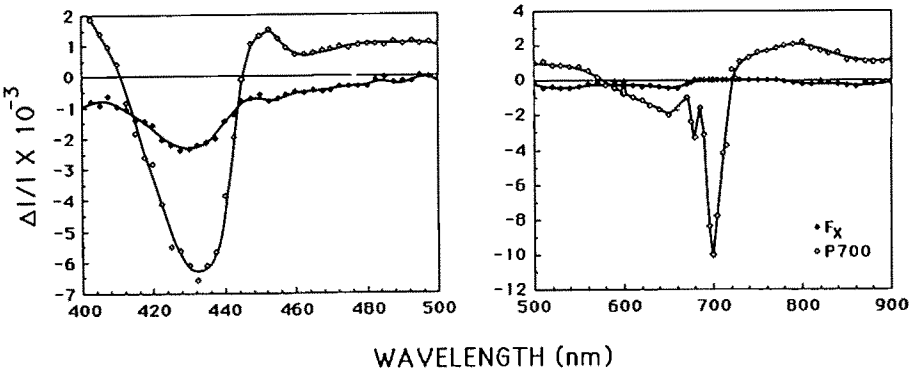


Fig. 5. Point-by-point difference spectrum of P-700 and $F_X$ in the Photosystem I core protein. The difference spectrum for P-700 (open diamonds) was obtained directly from a sample containing 0.033 mM DCPIP, 1.7 mM ascorbate and 2 mM methyl viologen. The absorption change for $F_X$ (closed diamonds) was obtained by subtracting the absorption change in a sample containing 0.033 mM DCPIP, 1.7 mM ascorbate and 2 mM methyl viologen from a sample lacking methyl viologen. The measurements were performed at 10 μg/ml chlorophyll and at 298 K.

P-700$^+$ and $F_X^-$ in the Photosystem I complex, when $F_A$ and $F_B$ have been prereduced, is 250 $\mu$s. We suggested earlier [8] that $F_A^-$ and $F_B^-$ may exert a coulombic effect on $F_X^-$ which could cause an acceleration in the backreaction time. This type of effect is not without precedent; a similar suggestion has been made for the 20 $\mu$s recombination of P-700$^+$ and $A_1^-$ in the presence of $F_X^-$ [23]. Since $[F_A/F_B]^-$ might also influence thermodynamic properties of $F_X$, we measured the midpoint potential of $F_X$ in the Photosystem I complex and in the Photosystem I core protein. Fig. 6 shows the amplitude of the P-700 absorption transient as a function of redox potential. The Photosystem I complex (Fig. 6a) follows a one-electron Nernstian equation (solid line) with a midpoint potential of $-670$ mV. For unknown reasons, this optically determined value is 35 to 40 mV higher than the $-705$ to $-10$ mV midpoint potential determined for $F_X$ in a similar preparation using low-temperature ESR techniques [24,25]. The Photosystem I core protein (Fig. 6b) also follows a one-electron Nernstian equation; however, the midpoint potential has shifted 60 mV to $-610$ mV. In addition to a possible Coulombic effect, the shift in potential might also occur if $F_X$ were exposed to a predominantly hydrophilic environment, rather than a hydrophobic environment. This might happen on removal of the low-molecular mass proteins, including the 8.9 kDa, $F_A/F_B$ polypeptide. Reconstitution experiments with a polypeptide containing an inactive chromophore may be required to decide between these two alternatives.

*ESR studies of the Photosystem I core protein*

Fig. 7a shows the light-minus-dark (before light) ESR spectrum of $F_X$ in the Photosystem I core protein when frozen in the dark and illuminated at 7.5 K. Since the core protein lacks $F_A$ and $F_B$, photoreduction of $F_X$ occurs without the need for strongly reducing conditions. Note the prominent resonance at $g = 1.78$ and 1.88 (the low-field resonance occurs too close to the $g = 2.0026$ resonance of P-700$^+$ to be clearly resolved). When the sample is warmed, illuminated at room temperature, and frozen to 7.5 K in the presence of continuous illumination, the spectrum is approximately twice as large as that obtained by illumination at 7.5 K and has a slightly altered line shape (Fig. 7b). The increase in magnitude does not derive from insufficient light intensity in the dark-frozen sample nor from a change in the microwave power saturation characteristics of $F_X$ (data not shown). Rather, an inherent heterogeneity in the low-temperature photoreduction of $F_X$ appears to be responsible for the difference in signal intensity.

Fig. 7c represents the difference spectrum obtained by subtraction of the sample illuminated at 7.5 K (Fig. 7a) from the sample frozen in the presence of continuous illumination (Fig. 7b). Although the magnitude of

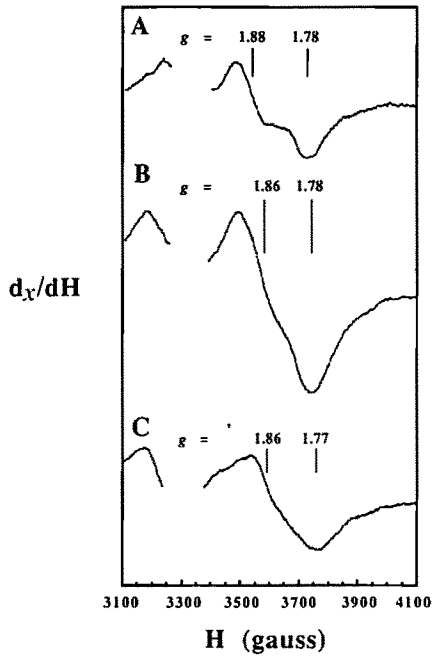


Fig. 7. ESR spectrum of $F_X$ in the Photosystem I core protein. (A) Light-minus-dark (before light) difference spectrum of $F_X$ in a Photosystem I core protein frozen in darkness and illuminated at 7.5 K. (B) Light-minus-dark (before light) difference spectrum of $F_X$ in a Photosystem I core protein illuminated during freezing. (C) The spectrum of $F_X$ frozen in the dark (A) subtracted from the spectrum of $F_X$ illuminated during freezing (B). The sample contained 500 $\mu$g/ml chlorophyll, 1 mM ascorbic acid and 0.8 mM DCPIP in 0.1 M Tris (pH 8.3). Spectrometer conditions: temperature, 7.5 K; microwave power, 40 mW; microwave frequency, 0.295 GHz; receiver gain, $2.5 \cdot 10^3$; modulation amplitude, 40 G at 100 kHz. The spectrum represents the average of four 4-min sweeps each for the light and dark samples, and a 10-fold enlargement in software.

the high- and mid-field resonances in Figs. 7a and 7c are approximately the same, the line shapes are significantly different and the $g$ values are shifted (particularly the midfield resonance at $g = 1.86$). For the purpose of this discussion, the $g = 1.86$, 1.77 species will be referred to as $F_{XH}$ (for high-temperature photoreducible) and the $g = 1.88$, 1.78 species will be referred to as $F_{XL}$ (for low-temperature photoreducible). This assignment is purely operational and does not necessarily imply that two distinct populations of $F_X$ are present under physiological conditions. Reduction of $F_{XH}$ is apparently achieved only by photoaccumulation at room temperature; increased illumination times for periods up to 2 h do not result in a progressively larger ESR signal of $F_{XL}$.

*Correlation between low-temperature optical and ESR studies*

The inefficiency in forward electron flow among the electron acceptors at low temperature should be reflected in the presence of at least two distinct kinetic phases of P-700$^+$ rereduction. One phase should represent the recombination between P-700$^+$ and $F_X^-$ while

the other phase should represent the recombination between P-700$^+$ and an earlier acceptor, either $A_0^-$ or $A_1^-$. Fig. 8 shows that the P-700$^+$ rereduction transient in the Photosystem I core protein at 77 K decays with the (expected) two distinct kinetic phases. The faster component, which constitutes 60% of the total absorption change, decays with a half-time of 800 $\mu$s, and represents the relaxation of the P-700 triplet state. The slower component, which constitutes the remaining 40% of the absorption change, decays with a half-time of 80 ms, and represents the recombination between $F_X^-$ and P-700$^+$. We suspect that the 80 ms, P-700$^+$ $F_X^-$ phase may correspond to those reaction centers containing $F_{XL}$, while the 800 $\mu$s, $^T$P-700 decay may correspond to those reactions centers containing $F_{XH}$. In the latter case, the electron backreacts with P-700$^+$, presumably at $A_0^-$, before reaching $F_{XH}$.

Careful comparison of Figs. 1 and 8 shows that the absorption change at 698 nm in the Photosystem I core protein is 1.6-fold larger at 77 K than at 296 K. The low-temperature difference sprectrum (Fig. 9) clearly indicates that the absorption change is due to P-700 photooxidation, but the main peak is located at 702 nm rather than 698 nm. However, at cryogenic temperatures, each band is sharper and more intense, and some of the wavelengths are shifted to the red (compare Figs. 5 and 9). The bleaching at 680 and 698 nm becomes more pronounced and it shifts to 680 and 702 nm at 77 K; the absorbance increases at 672 and 682 nm are also more pronounced and they shift to 674 and 688 nm at 77 K. At 702 nm, the absorption change is nearly twice as large as at 698 nm (at 296 K) (see also Ref. 26), an effect that may be due to the presence of a damaged reaction centers (and hence an underestimate of the P-700 absorption change at room temperature) but due

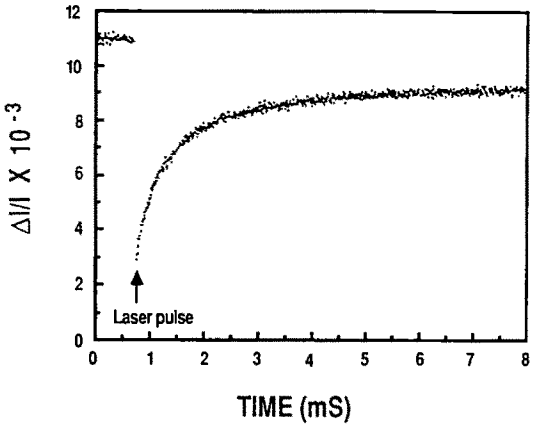


Fig. 8. Flash-induced absorption transient at 698 nm of the Photosystem I core protein at 77 K. The reaction mixture contained 5 $\mu$g/ml chlorophyll, 0.033 mM PMS and 1.7 mM sodium ascorbate. Excitation was provided by an 800 ps nitrogen laser. Note that an 800 $\mu$s phase constitutes approx. 60% of the total signal, representing inefficiency in forward electron transfer to $F_X$ and subsequent relaxation of the P-700$^+$ $A_0^-$ charge separation through the P-700 triplet state.

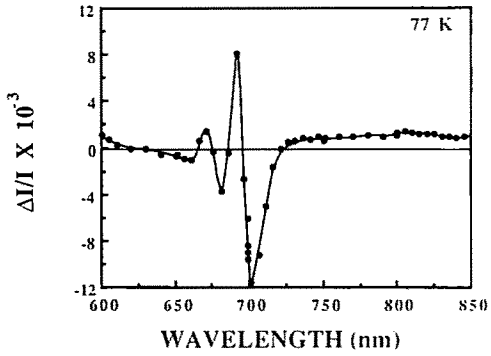


Fig. 9. Point-by-point difference spectrum of P-700 in the Photosystem I core protein at 77 K. The reaction mixture contained 5 $\mu$g/ml chlorophyll, 0.033 mM PMS and 1.7 mM sodium ascorbate. Excitation was provided by an 800 ps nitrogen laser.

more likely to the sharpening of the absorption bands at low temperature. Since the difference spectrum of P-700 is probably due to two different absorbance bands, one oxidized and one reduced [27,28], an increased absorbance at 688 nm at 77 K would be expected to result from a narrowing of each band at low temperature.

## Discussion

We have shown that urea is but one member of a large class of chaotropic agents that cause release of the low-molecular-mass polypeptides from higher plant and cyanobacterial Photosystem I reaction centers without destruction of iron-sulfur center $F_X$. The total loss of $F_A$ and $F_B$, the complete retention of $F_X$, the enhanced stability of the iron-sulfur cluster and the total lack of low-molecular-mass polypeptides differentiates this preparation from an earlier attempt using spinach reaction centers and LDS to obtain the intact Photosystem I core protein. We expect that this preparation will prove especially useful in studying the properties of $F_X$ without influence from $F_A$ and $F_B$.

The heterogeneity seen in $F_X$ in this preparation is problematic; at best, the low-temperature behavior of $F_X$ is reminiscent of $F_A$ and $F_B$. Previous low-temperature ESR experiments on a Photosystem I complex have indicated that only about 40% of $F_A$ is photoreduced after a single turnover flash and that subsequent flashes lead to a maximum of 67% of $F_A^-$ [28,29]. Similarly, the amplitude of the $F_X^-$ signal induced by illumination at 7.5 K is never more than 50% of the amplitude of the signal when $F_X$ is prereduced by illumination during freezing or by illumination at 210 K [30]. Optical experiments with digitonin Photosystem I particles at low temperature have shown that after chemical reduction of $F_A$ and $F_B$, electron transfer to $F_X$ and subsequent relaxation with P-700$^+$ occurs in only 10–15% of digitonin Photosystem I particles at low temperature; the majority of the backreaction occurs from $A_1^-$ to P-700$^+$ [28]. Mössbauer experiments also

indicate that only one-half of the iron in $F_X$ can be photochemically reduced at low temperature [32]. One explanation is that there exists heterogeneity within the population of $F_X$ such that only a portion of the reaction centers are active in electron transport at low temperature. The other possibility is that various conformations and distances between electron acceptors are frozen-in at low temperature, leading to a large range in reaction rates and yields. Taken together, these results indicate that the inefficiency in low-temperature photoreduction of $F_A$ and $F_B$ may be a consequence of a single locus located prior to, or at, iron-sulfur center $F_X$.

The presence of 4 mol of acid-labile sulfide in the Photosystem I core protein, the cysteine content of the *psaA* and *psaB* polypeptides, and the need for four cysteine ligands to bind both [2Fe-2S] and [4Fe-4S] clusters leads to a closed set of conditions: determination of the stoichiometry of the high-molecular-mass polypeptides should determine the cluster identity of $F_X$, and determination of the cluster identity of $F_X$ should specify the minimum stoichiometry of the high-molecular-mass polypeptides (assuming, of course, that $F_X$ is a traditional iron-sulfur cluster). The current assessment, based on uniform $^{14}C$-labeling data [33] and on volume calculations derived from electron-microscope-based dimensions [34], is that the Photosystem I complex consists of two, rather than four, high-molecular-mass polypeptides. We have observed that bifunctional cross-linking agents, which form covalent bonds with lysine residues, and SDS-PAGE under highly denaturing conditions, show a cross-linked band in the Photosystem I core protein with a molecular mass twice, rather than four times, that of the reaction center monomer. The combined stoichiometric data compels us to reconsider our previous conclusion that the Photosystem I core protein consists of four high-molecular-mass polypeptides [35] in favor of a two-polypeptide structure, in which $F_X$ is a [4Fe-4S] cluster ligated by homologous cysteine on the *psaA* and *psaB* proteins.

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## References

1 Malkin, R. (1987) in Photosynthesis: The Light Reactions (J. Barber, ed.), pp. 495–525, Elsevier, Amsterdam.
2 Cantrell, A. and Bryant, D.A. (1987) Plant Mol. Biol. 9, 453–468.
3 Golbeck, J.H. (1988) Biochim. Biophys. Acta 895, 167–204.
4 Green, B. (1988) Photosynthesis Res. 15, 8–32.
5 Interschick-Niebler, E. and Lichtenthaler, H.K. (1981) Z. Naturforsch. 36c, 276–283.
6 Takahashi, Y., Hirota, K. and Katoh, S. (1985) Photosyn. Res. 6, 183–192.
7 Schoeder, H.-U. and Lockau, W. (1986) FEBS Lett. 199, 23–27.
8 Golbeck, J.H. and Cornelius, J.M. (1986) Biochim. Biophys. Acta 849, 16–24.
9 Warden, J.T. and Golbeck, J.H. (1986) Biochim. Biophys. Acta 849, 25–31.
10 Golbeck, J.H., Parrett, K.G. and McDermott, A.E. (1987) Biochim. Biophys. Acta 893, 149–160.
11 Golbeck, J.H., Parrett, K.G., Mehari, T., Jones, K.L. and Brand, J.J. (1988) FEBS Lett. 228, 268–272.
12 Chua, N-H., Matlin, K. and Bennoun, P. (1975) J. Cell. Biol. 67, 361–377.
13 Hayashida, N., Matsubayashi, T., Shinozaki, K., Sugiura, M., Inoue, K. and Hiyama, T. (1987) Curr. Genet. 12, 247–250.
14 Oh-oka, H., Takahashi, Y., Wada, K., Matsubara, H., Ohyama, K. and Ozeki, H. (1987) FEBS Lett. 218, 52–54.
15 Høj, P.B., Svendsen, I., Scheller, H.V. and Møller, B.L. (1987) J. Biol. Chem. 262, 12676–12684.
16 Malkin, R., Aparicio, P.J. and Arnon, D.I. (1974) Proc. Natl. Acad. Sci. USA 71, 2362–2366.
17 Wynn, R.M. and Malkin, R. (1988) FEBS Lett. 229, 293–297.
18 Oh-oka, H., Takahashi, Y., Matsubara, H. and Itoh, S. (1988) FEBS Lett. 234, 291–294.
19 Oh-oka, H., Takahashi, Y., Kuriyama, K., Seaki, K. and Matsubara, H. (1988) J. Biochem. 103, 962–968.
20 Krantz, W.A. and Myers, J. (1955) Am. J. Bot. 42, 282–287.
21 Sétif, P., Quaegebeur, J.P. and Mathis, P. (1982) Biochim. Biophys. Acta 681, 345–353.
22 Golbeck, J.H., Lien, S. and San Pietro, A. (1977) Biochim. Biophys. Acta 178, 140–150.
23 Mathis, P. and Sétif, P. (1986) Photosyn. Res. 9, 47–54.
24 Warden, J.T. (1981) Biophys. J. 33, 264a.
25 Chamorovska, S.K. and Cammack, R. (1982) Photobiochem. Photobiophys. 4, 195–200.
26 Mathis, P., Sauer, K. and Remy, R. (1978) FEBS Lett. 88, 275–278.
27 Ikegami, I. and Itoh, S. (1988) Biochim. Biophys. Acta 934, 39–46.
28 Ikegami, I. and Itoh, S. (1988) Biochim. Biophys. Acta 851, 75–85.
29 Crowder, M. and Bearden, A. (1983) Biochim. Biophys. Acta 722, 23–35.
30 Bonnerjea, J. and Evans, M.C.W. (1984) Biochim. Biophys. Acta 767, 153–159.
31 Sétif, P., Mathis, P. and Vänngård, T. (1984) Biochim. Biophys. Acta 767, 404–414.
32 Evans, E.H., Dickson, D.P.E., Johnson, C.E., Rush, J.D. and Evans, M.C.W. (1981) Eur. J. Biochem. 118, 81–84.
33 Wynn, R.M. and Malkin, R. (1988) Biochemistry 27, 5863–5869.
34 Boekema, E.J., Dekker, J.P., Van Heel, M.G., Rögner, M., Saenger, W., Witt, I. and Witt, H.T. (1987) FEBS Lett. 217, 283–286.
35 Golbeck, J.H., McDermott, A.E., Jones, W.K. and Kurtz, D.M. (1987) Biochim. Biophys. Acta 891, 94–98.