

Tentative identification of the apoproteins of iron-sulfur centers of Photosystem I

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A newly purified Photosystem (PS) I particle is described, with still active iron-sulfur acceptors: A, B and X. Apart from the apoprotein of P700, 3 other main polypeptides of this particle are located at 20, 17 and 10 kDa, and two minor ones are detectable at 16.5 and 8 kDa. Both in vivo ^{35}S labeling and carboxymethylation with iodo[^{14}C]acetate show that most of the cysteine residues are located in the 8-kDa band. The amino acid composition of this band reveals important common features with small iron-sulfur proteins of the ferredoxin type.

<i>Photosystem I</i>	<i>Digitonin/deoxycholate particle</i>	<i>Iron-sulfur protein</i>	^{35}S labeling
	<i>Amino acid composition</i>	<i>Photosynthesis</i>	

1. INTRODUCTION

Extensive work has been done during the past decade aimed at purifying Photosystem (PS) I particles as simple as possible and still able to carry out the primary photochemistry, and to retain functional secondary acceptors: the Fe-S centers A, B and X [1]. These acceptors were mainly characterized by EPR and by flash absorption spectroscopy. In such particles, apart from the P700 apoprotein(s) at about 65 kDa, various polypeptides between 25 and 8 kDa are always present [2–9]. However, little is known about the precise correlation between these low- M_r polypeptides and electron donors or acceptors [10,11]. This may be due to the fact that the drastic conditions required to extract them from the PS I complex damage the clusters [12]. It has been claimed that some iron was associated with polypeptides of about 15 and 18 kDa in a purified PS I from barley [13]. However, most of the low-potential Fe-S proteins of the ferredoxin type characterized so far have lower molecular mass (between 6 and 10 kDa) especially those with 4Fe-4S clusters [14]. Moreover, radioactive iron has been recovered in a protein fraction of about 7 kDa in a PS I from a

Cyanobacterium [15]. This prompted us to reassess these preceding assignments, and this work will try to determine, essentially by the mean of biochemical experiments, which among the associated polypeptides are likely to be the Fe-S acceptors of the PS I.

2. MATERIALS AND METHODS

Spinach PS I particles were prepared by deoxycholate electrophoresis following digitonin solubilization as in [16]; this particle is further designated as nPS I (normal PS I). A somewhat different particle, PS I β , was prepared in the same way except that the digitonin step was preceded by overnight incubation of the thylakoid membranes in the presence of 5% β -mercaptoethanol at pH 8.8. This treatment is a way to destabilize the membrane structure and was designed to obtain a deeper action of the detergent with the eventual release of non-essential proteins.

For in vivo labeling experiments, 10 young spinach leaves were cut and fed either with 0.2 mCi ^{59}Fe as the citrate salt (32 mCi/mg), or with 4 mCi ^{35}S as inorganic sulfate (25–40 Ci/mg). Absorption of the aqueous solution (100–200 μl per leaf)

took about 2 h under strong illumination; leaves were then transferred to water under normal illumination for 48 h. Preparative and analytical electrophoresis was done as in [16]. Amino acid analyses were performed after 24 h hydrolysis under vacuum in the presence of an azeotropic HCl/water mixture; when needed for accuracy, thioglycollic acid was added as a protective agent for a better recovery of methionine. Labile sulfide was estimated following a modification [17] of the method in [18]. Acid-soluble iron [18] was measured by atomic absorption spectrometry using a 290B Perkin Elmer apparatus.

The amount of P700 was measured by the chemical difference spectrum and by the flash-induced absorption change on the millisecond time scale [19], taking a $\Delta\epsilon$ of $6.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for P700^+ at 820 nm [20].

EPR measurements were made using a Bruker ER 200 tt equipped with an Oxford Instruments ESR 900 liquid helium apparatus. The samples were frozen under illumination in the presence of 20 mM dithionite at pH 10.

3. RESULTS AND DISCUSSION

The new particle PS I β has been used throughout this work. The Chl:P700 ratio, as estimated by the chemical difference spectrum or by the flash-induced absorption change at 820 nm, is about 50–60, which is half the ratio of nPS I. This ratio is very close to that of CP1 [21], a highly purified P700 reaction center no longer with Fe-S centers. In the case of PS I β , the amounts of acid-labile sulfide and iron are unchanged with respect to nPS I [7]: mean values are 14 and 10–12 per P700, respectively, which is compatible with the presence of three 4Fe-4S centers; the absence of cytochromes was previously shown by spectroscopy and specific staining after gel electrophoresis [7]. Low-temperature EPR corroborates the presence of the 3 acceptors of PS I: centers A, B and X (fig.1). At room temperature, the flash-induced absorption change at 820 nm relaxes slowly ($t_{1/2} \gg 1 \text{ ms}$), thus indicating that these acceptors are functional [19,22].

Upon analytical SDS electrophoresis (fig.2), PS I β dissociates into CP1 (the chlorophyll-protein complex at about 110 kDa), and essentially 3 low- M_r polypeptides. The main protein of CP1

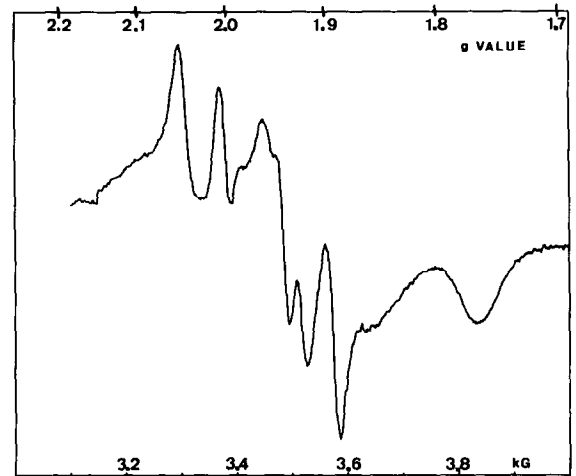


Fig.1. EPR spectrum of PS I β particles under highly reducing conditions. The particles were frozen under illumination in the presence of 20 mM sodium dithionite, 0.2 M Tris buffer (pH 10). Instrument settings: microwave power, 20 mW; modulation, 20 G; temperature, 10 K. Chlorophyll concentration was 0.5 mg/ml.

observed after heat dissociation of the chlorophyll complex is a diffuse band at 62/65 kDa (see fig.3, lane A2); this band was found more or less heterogeneous by different authors and has been the subject of a detailed discussion [23]. In the case of CP1 from spinach, the components at 62 and 65 kDa were previously shown, by limited proteolysis, to be tightly related proteins [24]. This result has been confirmed more recently and extended to other organisms [25,26]. The 3 other main components are located at about 20, 17 and 10 kDa; a faint band just below the 17-kDa polypeptide (16.5 kDa) and a small diffuse or heterogeneous band at 8 kDa are also observed. The latter is more heavily stained after preliminary fixation in the gel by incubation with glutaraldehyde (5%, 1 h). A similar pattern, with 3 or more polypeptides in the 25–8 kDa range, has been widely observed in different well purified PS I particles [2–9]. Authors in [8], working with an apparently more stable complex from a thermophilic cyanobacterium, found that only low-molecular mass components around 10 kDa seemed to be involved in the electron transfer to P430.

From comparison with the electrophoretic pattern of nPS I, we can see that peptides above

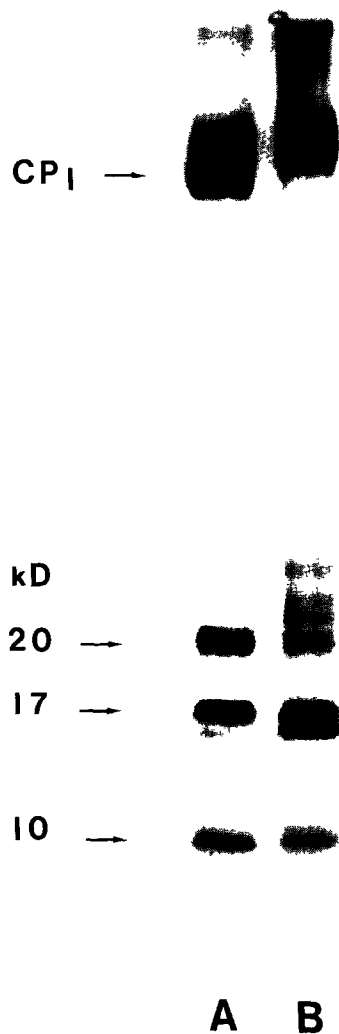


Fig.2. Polypeptide composition of PS I β (A) and nPS I (B) as observed by analytical SDS-PAGE. The running gel was 15% acrylamide, 0.125 M Tris-HCl (pH 8.5), 0.1% SDS, with a ratio of acrylamide to bisacrylamide of 120. A prerun of the gel allowed a good separation of the low- M_r components. SDS was added to each sample just prior to electrophoresis at a final SDS:chlorophyll ratio of 10.

20 kDa are no longer present in PS I β (fig.2); a similar modification was shown by authors in [5] to occur during the transformation of their PS I 110 into PS I 65, which they attributed to the loss of a peripheral antenna. Moreover, in PS I β , the 17-kDa band becomes far more significant than the 16.5-kDa band, in contrast to nPS I.

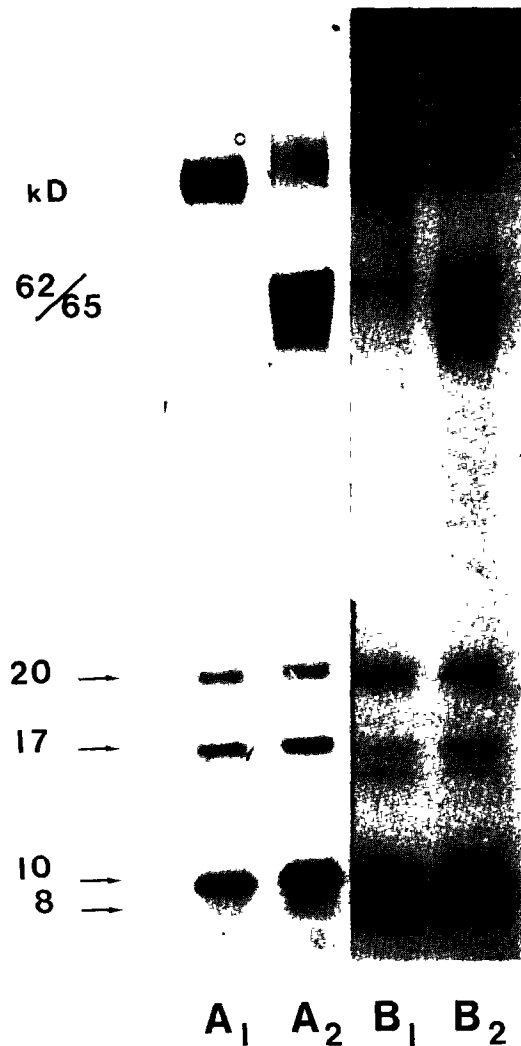


Fig.3. Stained gel and autoradiography of ³⁵S-labeled PS I β ; electrophoretic conditions were the same as described in fig.2. Lanes A: patterns observed after Coomassie blue staining, without any treatment (A₁) and after heat dissociation, 30 s at 80°C (A₂). Lanes B: the same samples as observed by autoradiography.

The problem of Fe-S protein identification was first examined by incorporation of ⁵⁹Fe. It was supposed that Fe-S clusters were stable during denaturing electrophoresis in the presence of SDS. This appeared not to be the case, and no radioactive band was found, even using lithium dodecyl sulfate electrophoresis in the cold. A similar result has been observed for the PS I of *Mastigocladus laminosus* for which iron remains associated only

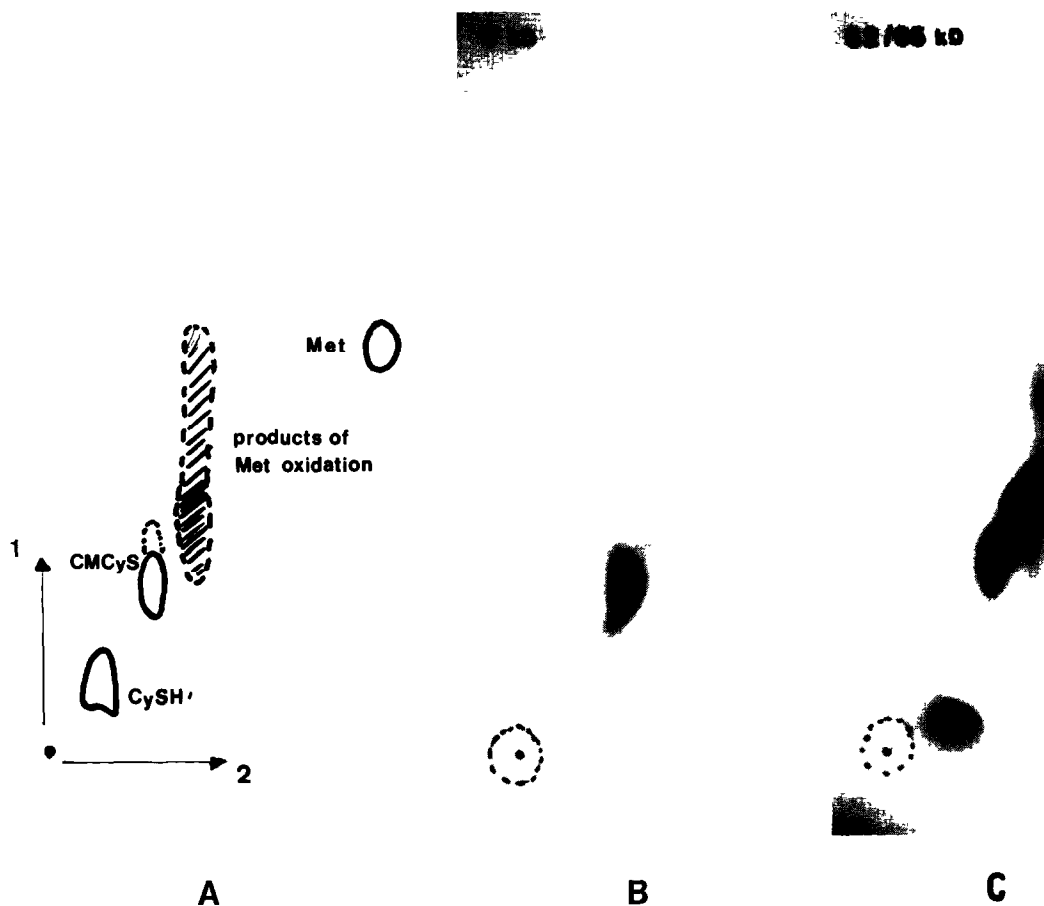


Fig.4. Bidimensional thin-layer chromatography of amino acid mixtures on cellulose plates. Solvent 1: butanol, acetic acid, water (70:10:25, v/v); solvent 2: pyridine, isoamyl alcohol, water (35:35:30, v/v). (A) Schematic representation of a standard mixture of cysteine, carboxymethylated cysteine (CMCys), methionine (Met) and methionine oxidation products resulting from acid hydrolysis. (B) Autoradiography of the amino acid mixture from acid hydrolysis of ³⁵S-labeled 8-kDa band. (C) Autoradiography of the amino acids from the 62/65-kDa band in the same conditions.

in the native complex [27]. We then took opportunity of the fact that candidates for small Fe-S proteins would have a high content of cysteine residues (4–8 for 10 kDa), and thus would be strongly labeled after *in vivo* ³⁵S incorporation. After such an experiment, fig.3 shows that among the small proteins of PS I β , most of the radioactivity is located in the 8-kDa band, contrasting with its poor staining. The double band at 62/65 kDa is also heavily labeled, with 50% of the total activity instead of 35% for the 8-kDa component. This last figure cannot be due to the presence of sulfolipids as the lipidic moiety migrates faster and cannot account for more than 5% of the total activity, as checked by organic extractions. To

discriminate between the radioactivity of methionine and cysteine, we subjected a large amount of ³⁵S-labeled PS I β to preparative electrophoresis. Prior carboxymethylation of the proteins was performed, which allowed a better staining of the 8-kDa band. Both the 62/65- and 8-kDa regions were extracted, digested by acid hydrolysis, and the resulting amino acid mixtures subjected to separation by thin-layer bidimensional chromatography. Fig.4 shows that the only radioactive spot for the 8-kDa component is carboxymethylated cysteine, which is not the case for the 62/65-kDa bands where most of the activity has been incorporated into the methionine (detected as its oxidized products). This experiment argues for the

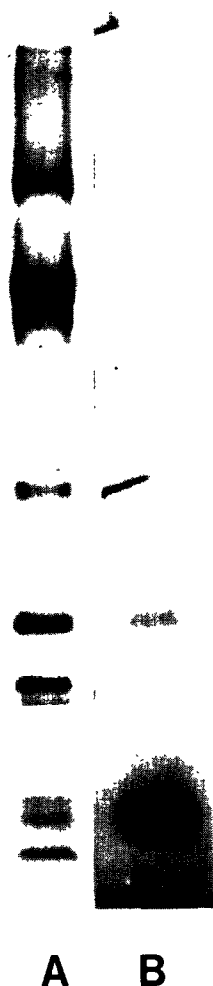


Fig.5. Stained gel (A) and autoradiography (B) of a sample of PS I β after carboxymethylation of the cysteine residues by iodo[^{14}C]acetate. The sample was first reduced overnight at 4°C by 1% mercaptoethanol in 8 M urea, 0.4 M Tris-HCl buffer (pH 8.8); iodoacetate was added in a slightly smaller amount than mercaptoethanol. Electrophoresis was as described in fig.2.

8-kDa band as being the peptide part of some Fe-S acceptors of PS I. The small relative amount of cysteine in the 62/65-kDa components remains nevertheless compatible with a site for an Fe-S cluster, as only 2 cysteine residues are required as ligands, even in a large protein. Moreover, the equivalent of less than one atom of iron remains systematically associated with the CP1 particle, which is only constituted of the 62/65-kDa components [24]. This failure to remove the iron

entirely has been observed for high- M_r Fe-S proteins [28]. We thus cannot definitely exclude that, for instance, the more primary acceptor, X, would be an integral part of the 62/65-kDa protein, despite a clear identification as the Fe-S cluster is still lacking [24].

Carboxymethylation of PS I β in a denaturing medium (8 M urea) using iodo[^{14}C]acetate led to the same conclusion as ^{35}S labeling. The reaction was performed under conditions strictly specific for cysteine residues [29]. Fig.5 clearly confirms that the main component with a high amount of carboxymethylated cysteine is located at 8 kDa. Only minor labeling was observed at the level of the 20-kDa band, and practically none in the region of the 62/65-kDa components. The latter point shows the poor accessibility of the few cysteine residues of this protein, even in 8 M urea.

The amino acid composition of this 8-kDa component is given in table 1. One can notice the high level of acidic residues and carboxymethylated cysteines (CMCyS), and the presence of just two aromatic amino acids. In most common low- M_r Fe-S proteins [14], Fe-S clusters are linked to the

Table 1
Amino acid composition of the 8-kDa band obtained after 24 h hydrolysis under vacuum

Amino acid	Mol%	Minimum number of residues
CMCyS	9.6	7
Asp (+ Asn)	7.7	5
Thr	6.2	4
Ser	7.3	5
Glu (+ Gln)	10.2	7
Pro	7.6	5
Gly	10.1	7
Ala	8.3	6
Val	6.6	4
Met	1.7	1
Ile	4.1	3
Leu	5.5	4
Tyr	1.4	1
Phe	1.7	1
His	1.6	1
Lys	5.2	4
Arg	4.7	3

Each value is the mean of 3 determinations starting with 3 different preparations

peptidic backbone by 4 cysteines, and they often interact with an aromatic residue, all details which are compatible with the composition shown here. The calculated M_r is about 7500 (tryptophan was not determined). This result is in good agreement with an early estimation by authors in [30] working directly on the whole thylakoid membrane, and with that of authors in [15] concerning a sulfur-rich polypeptide associated with the PS I of *Anacystis nidulans* R2 [15]. The high level of cysteine residues in our case is also compatible with the presence of two Fe-S clusters on the same polypeptide, as for ferredoxins of the anaerobic type [14].

We tried unsuccessfully to extract specifically the 20, 17 and 10-kDa components from PS I β using various chaotropic agents and detergents. In an experiment using 1 M sodium thiocyanate in 1% Deriphat, we obtained a particle with only CP1 and the 20- and 8-kDa components, and still having a normal EPR spectrum; but samples with a decreased amount of the 20-kDa component exhibited a concomitant non-specific decrease of all the EPR signals. All happens as if the 20-kDa polypeptide were involved in the cohesion of a functional PS I, as was postulated [25] following experiments with inhibitors of the protein synthesis.

Investigations are in progress to determine the exact number of peptide chains in the 8-kDa band, and eventually their sequences. This may help in determining whether centers A and B are located on the same or on two different protein moieties.

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