Evidence for a hetero-oligomeric structure of the chloroplast cytochrome b-559

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A second nonhomologous polypeptide in the thylakoid membrane cytochrome b-559 has been suggested by the finding of a smaller reading frame just slightly downstream from that corresponding to the 9 kDa cytochrome polypeptide that is dominant on a Coomassie-stained gel. This reading frame encoded a 39-residue polypeptide that was similar in having a central hydrophobic domain of 25–26 residues and a single His residue at the same position in the hydrophobic domain. The smallest polypeptide seen on SDS gels of the cytochrome was isolated by high-performance liquid chromatography (HPLC). The NH_2-terminal sequence matched that of the downstream gene. The stoichiometry of the 2 gene products separated by HPLC was approx. 1:1, based on the molecular masses of 9.16 and 4.27 kDa calculated from the nucleotide sequence. It is concluded that the cytochrome contains both the 9.16 kDa (α) and 4.27 kDa (β) polypeptides. These data, the single His residue on each polypeptide, and the previous finding of a bis-histidine coordination, imply that the unit heme binding structure of the cytochrome is a heme cross-linked dimer. If the cytochrome contains a single heme, the dimer structure would be ($\alpha\beta$). If there are 2 hemes/cytochrome, the more likely structure would be ($\alpha\beta)_2$, a tetramer consisting of 2 heme cross-linked hetero-dimers.

Cytochrome b-559   Amino acid sequence   Protein structure   Thylakoid membrane

1. INTRODUCTION

The chloroplast high-potential cytochrome b-559 is an intrinsic membrane protein of the chloroplast oxygen-evolving complex whose function is as yet enigmatic [1]. The purification of a small 9 kDa polypeptide [1,2] indicated that the structure of this protein might be relatively simple. More recent data suggested a more complicated structure [3–5], and the studies discussed here document that the cytochrome contains a second smaller polypeptide.

2. MATERIALS AND METHODS

Cytochrome b-559 was purified from spinach thylakoid membranes as in [1,5], and the putative

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cytochrome subunits separated by HPLC using a water-acetonitrile non-linear gradient as described in [1]. The NH_2-terminus of the 4.27 kDa cytochrome subunit was apparently blocked and would not yield sequence information until unblocked with 5% methanolic HCl for 24 h at room temperature [6]. Determinations of amino acid composition and NH_2-terminal sequence were carried out as in [1].

3. RESULTS

The cytochrome b-559 gene in the spinach plastic chromosome was located by hybrid selection-translation and coupled transcription-translation using appropriate recombinant DNAs and immunoprecipitation [3]. The antibody was made to the dominantly stained 9 kDa polypeptide of the cytochrome b-559 from spinach thylakoid

membranes, for which a 27-residue NH_2-terminal sequence has been determined [1]. The reading frame of the gene was found to code for an 83-residue polypeptide and, confirming the amino acid composition [1], to contain a single His residue as a possible heme ligand [4]. Unexpectedly, the TAG termination codon ending at base 252 was found to overlap with a possible ribosome binding sequence GGAGG, and a second reading frame was found to start just 5 bases further downstream with an ATG codon [4]. The second reading frame corresponded to a 39-residue polypeptide, again containing a single His residue. The His residue in both the 83-amino-acid reading frame and the downstream gene corresponding to 39 residues were located in the same relative positions within the translated protein sequences, 5 residues from the N-terminal side of a 25- or 26-residue domain of non-polar amino acids. The proximity of the downstream reading frame, the existence of the intervening putative ribosome binding site, the similar extent of a single hydrophobic domain in the 2 polypeptides, and the identical position of a single His residue in the hydrophobic domains of each polypeptide had suggested that the downstream reading frame might code for a second polypeptide component of the cytochrome b-559 protein [4]. It has also been determined that both reading frames are highly conserved in tobacco and *Oenothera* (Carrillo and Herrmann, unpublished).

Two polypeptides of 6 and 8 kDa, in addition to the predominant 9 kDa band, had been identified in the gels of the purified protein and were further purified by reverse-phase HPLC [1]. The actual M_r of the band of M_r 9000, from which the NH_2-terminal methionine has been removed by post-translational processing, is 9162 [4]. Although the 6 kDa component stained weakly on SDS-urea gels, the amount of this polypeptide relative to the 9 kDa polypeptide was seen to be larger when areas under the HPLC elution profile were compared ([1]; fig.1). The amino acid composition of the HPLC-purified 6 kDa component showed that it contained a single His residue and that there was no residue for which there was a difference greater than one when compared to the composition of the 39-residue polypeptide encoded by the downstream reading frame (table 1). In fact, after deblocking the N-terminal residue (see sec-

Table 1

Amino acid composition of the 4.27 kDa polypeptide (A) and that predicted from the nucleotide sequence (B)

	mol/mol His	
	A^a	B
Ala	3.4	3
Arg	3.2	3
Asx	1.6	1
Cys	n.m.	0
Glx	2.4	2
Gly	2.7	2
His	0.9	1
Ile	4.7	5
Leu	3.2	3
Lys	0.4	0
Met	0.9	1.0^b
Phe	3.7	3
Pro	2.0	2
Ser	2.0	3
Thr	3.2	4
Tyr	0.9	1
Tryp	n.m.	1
Val	3.1	3

n.m., not measured; [a] average of 2 determinations; [b] not including NH_2-terminal methionine

tion 2), the sequence of the 6 kDa polypeptide was found to match that predicted from the nucleotide sequence in 6 of the first 9 residues, with the NH_2-terminal methionine again removed by post-translational processing (table 2). The predicted amino acids were also present in positions 3 and 5 in the sequence shown in table 2, but one other residue was found in each of these cycles. The

Table 2

Comparison of (A) NH_2-terminal amino acid sequence of the isolated small polypeptide and (B) the sequence predicted for the 4.27 kDa product of the downstream reading frame

Measured

NH_2...-Thr-Ile-(Asp)-X-(Thr)-Tyr-Pro-Ile-Phe-...COOH

Predicted from nucleotide sequence

NH_2...-Thr-Ile-Asp-Arg-Thr-Tyr-Pro-Ile-Phe-...COOH

residue in position 4 could not be determined. These data show that the polypeptide product of the downstream gene is present in the purified cytochrome b-559 preparation. The actual M_r of the band originally described as M_r 6000 is 4268 without the N-terminal methionine [4]. The ubiquitous presence of this low-M_r band in gels of cytochrome b-559 purified from PS II particles of maize had been noted [2]. Not only is the 4.27 kDa downstream gene product present in gels containing cytochrome b-559 from spinach thylakoid membranes [1], but the stoichiometry of this component is approx. 1:1 with the 9.16 kDa polypeptide, as judged by the area underneath the 280 nm absorbance bands in the HPLC elution profile of the cytochrome (fig.1). Weighting the areas for the 3 Tyr and 2 Trp residues in the larger polypeptide, and the one Tyr and one Trp in the smaller [4], the stoichiometry was 1.07 in the experiment shown in [1], 1.35 in the HPLC elution profile of fig.1, and an average of 1.3 in 7 determinations.

From the comparison of the gene and polypeptide sequences and the relative stoichiometry of the 4.27 and 9.16 kDa polypeptides in the purified protein, it seems likely that at least one copy of
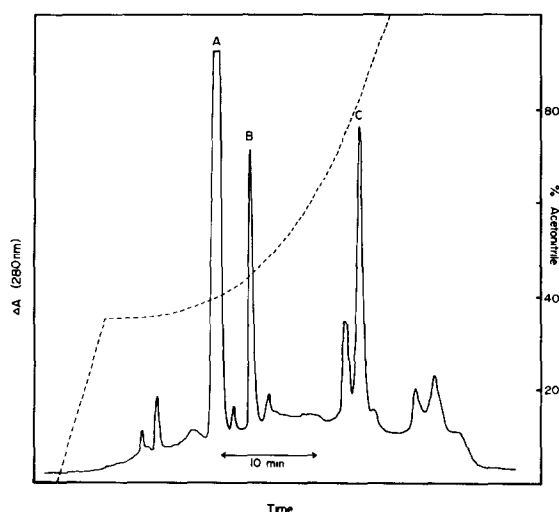

Fig.1. Separation by reverse-phase HPLC of the polypeptide components of cytochrome b-559 purified according to [1]. The elution pattern resulted from a continuous non-linear acetonitrile-H₂O gradient [1] that is shown (– – –). Elution bands A–C contain free heme, the 4.27 kDa, and the 9.16 kDa polypeptides, respectively, determined by gel electrophoresis as in [1].

each is present in the cytochrome b-559 protein, so that by these criteria the protein is at least a dimer since it must contain one copy of each polypeptide. The conclusion that the heme binding unit must also be dimeric had been reached (i) from the previous findings mentioned above that both 9.16 and 4.27 kDa polypeptides contained only one His residue [1,4], and (ii) that the heme coordination was found to be bis-histidine from EPR, optical and Raman spectroscopic studies [5]. Such a structure with heme cross-linking of separate polypeptides has not been previously found for any other soluble or membrane-bound cytochrome, although there is some similarity in the intramolecular heme cross-linking of 2 different membrane-spanning domains of the chloroplast cytochrome b_6 and the b cytochrome of the mitochondrial b-c_1 complex [7–9].

4. DISCUSSION

If cytochrome b-559 contains a single heme, then it follows from the bis-histidine coordination, the single His residue in the sequences of the 4.27 and 9.16 kDa polypeptides, and the approx. 1:1 stoichiometry of these polypeptides that the simplest cytochrome b-559 structural unit is an $(\alpha\beta)$ dimer (fig.2). A principal feature of the models shown in fig.2 is that the His residue in both α and β polypeptides is located in the hydrophobic domain of 25–26 residues and is 5 residues from a charged Arg residue that borders these domains on the NH₂-terminal side. The models assume that the non-polar domain spans the hydrophobic membrane core in an α-helical conformation. There is a substantial [7,10,11], though not a necessary [12], precedent for the latter assumption.

The possibility that the cytochrome may, in fact, be a polypeptide tetramer arises from several experiments indicating that cytochrome b-559 contains 2 hemes. (i) Although there is disagreement in the literature over the total number of b-559 hemes per P700 or cytochrome f [13–15], related to the disagreement over the existence and location of a low-potential cytochrome b-559 [14], it is generally agreed that there are 2 high-potential b-559 hemes per reaction center in thylakoid membrane preparations [13,14]. (ii) Spectroscopic data indicate that one b-559 heme can be photooxidized at
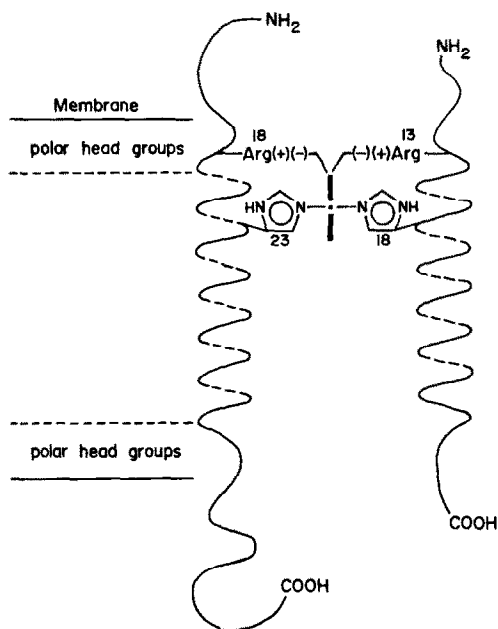
Fig.2. Models for folding of a monoheme unit of cytochrome *b*-559 in the hydrophobic core of the thylakoid membrane. It is assumed that the central 25- or 26-residue non-polar domain of both α and β polypeptides spans the hydrophobic core of the membrane in an α-helical conformation. The latter assumption is based on the similarity between the thickness (30–40 Å) of this membrane core and the length of 20–25 residues in an α-helix, as well as the precedents of the chloroplast cytochrome b_6 and the mitochondrial *b* cytochrome of complex III, where a similar model for a transmembrane arrangement of the 2 hemes seems to apply as well [7–9].

77 K [16], and one heme can be efficiently ($t_{1/2}$ = 100 ms) photoreduced at room temperature [17], suggesting a function of one heme on each side of the thylakoid membrane [18]. (iii) Consistent with the latter hypothesis is the biphasic oxidation by the membrane-impermeable oxidant, ferricyanide, of 2 high-potential cytochrome *b*-559 hemes [19]. The finding of one heme per P680 reaction center (e.g. [20,21]), instead of 2 (e.g. [22,23]), in PS II particle preparations could be explained by different heme environments on the 2 sides of the membrane, and greater lability of one heme leading to its loss from the membrane. The 2 different environments proposed for the 2 cytochrome hemes, one close to the stromal, one to the lumenal interface, might also explain the

greater lability toward conversion to lower potential of approximately half of the cytochrome *b*-559 population [17,22,23].

A model for cytochrome *b*-559 containing 2 hemes could consist of 2 oppositely oriented heme cross-linked ($\alpha\beta$) heterodimers, an ($\alpha\beta$)$_2$ polypeptide tetramer, with the hemes on opposite sides of the central hydrophobic domain. In this model, as well as that of the ($\alpha\beta$) dimer, the predicted specific heme content, 1 heme per 14 kDa, is close to that previously measured, ~1/17 kDa [1]. An argument against this tetramer model is that the 2 identical heterodimers should not orient with opposite polarity in the membrane. However, the *b*-559 protein could be inserted into the membrane as a tetrameric unit. An (α_2)(β_2) tetramer model of 2 homodimers for a 2-heme cytochrome *b*-559 is less likely, since the α and β polypeptides have a net charge at neutral pH of +2 and −3, respectively. The α_2 and β_2 dimers would then differ by 10 charge units, as well as by 10 kDa in molecular mass, and should have been separable on the detergent-containing DEAE columns used in the purification of the protein.

It is of interest in the context of the problem of chloroplast gene evolution to note that the cytochrome *b*-559 gene may be a split gene, as has been proposed for the chloroplast cytochrome b_6 [7]. The observation of a cytochrome *b*-559 14 kDa polypeptide in SDS-PAGE gels of membranes from the green alga *Chlamydomonas reinhardi* [24], and a similar cross-reacting polypeptide in such gels of membranes from the cyanobacterium *Anacystis nidulans* (H. Pakrasi and L. Sherman, personal communication), suggests that the 2 *b*-559 genes may be fused in these older photosynthetic organisms.

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