N-terminal amino acid sequence of the Rieske iron-sulfur protein from the cytochrome b_6/f-complex of spinach thylakoids

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Received 5 August 1986

The 20 kDa subunit of the cytochrome b_6/f-complex, the Rieske iron-sulfur protein, was isolated by preparative SDS-PAGE. For automatic Edman degradation the protein was recovered by electrophoretic elution. Several peptides were purified after tryptic, CNBr or Arg C endopeptidase digestion or cleavage at Trp-residues. The determination of the amino acid sequence of these peptides yielded overlapping sequences for a total of 96 amino acids starting at the N-terminus. This sequence shows no homology to the primary structure of the two Rieske FeS proteins already published by other authors. But like these it contains a hydrophobic sequence of 30 amino acids close to the N-terminal indicating the sequence that spans the membrane. Two polypeptides, closer to the C-terminus, contained sequences which are also present in *Neurospora crassa* and *Rhodopseudomonas sphaeroides* including a Cys-Pro-Cys containing sequence involved in iron binding.

Chloroplast *Cytochrome* b_6/f-*complex* *FeS protein* *Amino acid sequence*

1. INTRODUCTION

The plastoquinol-plastocyanin oxidoreductase or cytochrome *b*-563/*f*-complex is an integral complex in the photosynthetic electron flow system in the thylakoid membrane of chloroplasts and connects photosystems I and II. The complex has been purified by Hurt and Hauska [1] and shown to consist of five subunits of 35, 34, 23, 20 and 17 kDa. According to these authors the two large subunits are cytochrome *f*, the 23 kDa subunit is cytochrome b_6, the 20 kDa subunit represents the Rieske FeS center and the smallest subunit is probably part of the cytochrome b_6 function. The primary protein structure of cytochrome *f* [2], cytochrome b_6 [3,4] and the 17.5 kDa subunit [4] are already known from DNA sequencing of the chloroplast genome. Neither DNA nor amino acid sequence of the nuclear coded FeS protein of a higher plant is so far available. Recently the gene for the Rieske FeS center of *Neurospora crassa* [5] and of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* [6] has been obtained. The N-terminal sequence of the Rieske FeS protein from spinach, determined by protein sequencing, is described here.

Abbreviations: PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl; N-terminal, amino-terminal; Mes, 2-(*N*-morpholino)ethanesulfonic acid; kDa, kilodalton; CNBr, cyanogen bromide

2. MATERIALS AND METHODS

Dithiothreitol was obtained from Serva. Acetonitrile, sodium hydroxide and mercap-

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00145793/86/$3.50

toethanol were from Roth. Trifluoroacetic acid and ethanethiol were purchased from Pierce. Phenylisothiocyanate was from Beckman. All reagents for sequence analysis were from Applied Biosystems. All other chemicals or solvents were of the highest purity available.

Iodo[^{14}C]acetic acid was delivered by Amersham Buchler.

2.1. *Purification of the cytochrome* b_6/f-*complex*

The cytochrome b_6/f-complex was isolated according to the procedure of Hauska [7] using spinach from the local market. It consists of five proteins of 35, 34, 23, 20 and 17 kDa as described. Its enzymic activity was measured in a dual wavelength spectrophotometer as cytochrome *c* reduction at 550/540 nm. The reaction mixture contained 10 μM cytochrome *c*, 1 μM plastocyanin, 50 μM 2,5-di-*tert*-butyl-1,4-hydroquinone and 50 nM cytochrome b_6/f-complex (measured via its cytochrome *f* content) in 25 mM Mes buffer, pH 6.5. The rates were in the range of 3–6 μmol cytochrome *c* reduced/h per nmol cytochrome *f*.

2.2. *Isolation of the Rieske FeS protein from the cytochrome* b_6/f-*complex*

The Rieske FeS protein was obtained either as described by Hurt et al. [8] or by electrophoretic elution of the protein band after separation with SDS-PAGE [9]. The FeS protein recovered by both methods was suitable for high-sensitivity amino acid analysis or automated amino-terminal sequence analysis. The purity of the protein was tested by analytical SDS-PAGE, showing a single band with the original electrophoretic mobility. In order to prevent amino-terminal blockage during the preparative PAGE [10] application of urea was avoided and 0.1 mM sodium thioglycolate was added to the cathode buffer reservoir. The Rieske FeS polypeptide obtained according to Hurt et al. [8] contained 1.7 atoms Fe per molecule protein. Fe was determined by the method of Brumby and Massey [11]. The protein electroeluted from the SDS gel contained no iron. The protein was modified by iodoacetic acid using iodo[^{14}C]acetic acid [12].

2.3. *Tryptic digestion*

Cleavage by trypsin (2%, w/w) was performed for 16 h at 37°C in 0.05 M ammonium bicarbonate solution, pH 8.0. The fragments were separated by HPLC on a reverse-phase RP-18 column in 0.1% trifluoroacetic acid with an acetonitrile gradient ranging from 0 to 60%.

2.4. *CNBr treatment*

5 mg FeS protein dissolved in 1 ml of 70% formic acid was cleaved with 14 mg CNBr (500-fold molar excess over methionine) at 30°C for 18 h. The reaction was carried out under nitrogen in a 16 × 95 mm screw-cap tube. Then the formic acid was removed by evaporation and the sample was three times redissolved in destilled water and lyophilized before preparative SDS-PAGE.

2.5. *Combined cleavage at Met and Trp residues [13]*

5 mg FeS protein was dissolved in a mixture of 0.5 ml of 88% formic acid and 0.5 ml anhydrous heptafluorobutyric acid. After addition of 700 mg of solid CNBr the mixture was held at 25°C in the dark for 24 h. The reagent and the solvents were removed with a stream of nitrogen. The remaining material was three times suspended in 10 ml water and lyophilized before preparative SDS-PAGE.

2.6. *Amino acid analysis*

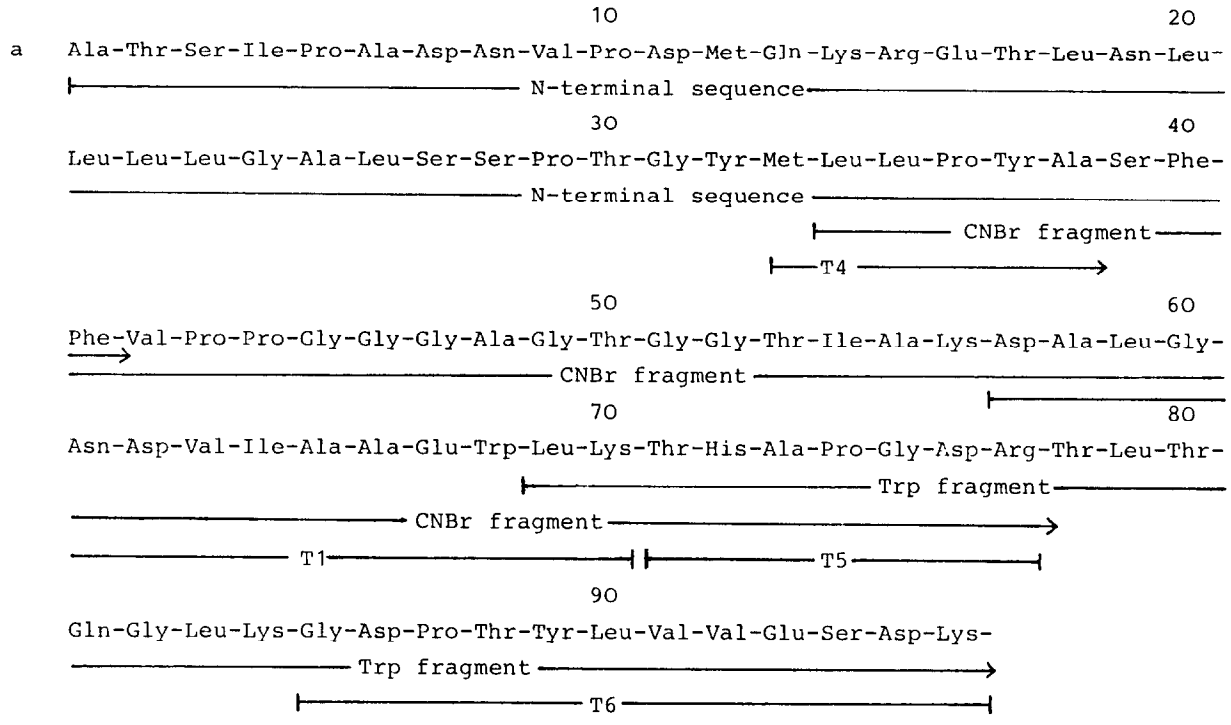
Samples were hydrolyzed in 6 M HCl at 150°C for 1 h. The analysis was done by precolumn derivatization with phenylisothiocyanate and reversed-phase HPLC of the resulting PTC-amino acids [14,15].

2.7. *Sequence analysis*

The amino acid sequence was determined by automated gas-phase Edman degradation with online identification of the PTH amino acids [16].

3. RESULTS AND DISCUSSION

The Rieske FeS protein was either isolated from a purified cytochrome b_6/f-complex by the method of Hurt et al. [8] by hydroxyapatite and DEAE column chromatography or by electrophoretic elution of the 20 kDa band after SDS-PAGE. Both preparations were characterized by N-terminal sequencing that yielded identical sequences (fig.1a). This dual sequencing was done to ensure that the



b T1: Asp-Ala-Leu-Gly-Asn-Asp-Val-Ile-Ala-Ala-Glu-Trp-Leu-Lys (residue 57-70)

T2: Val-Val-Phe-Val-Pro-Trp-Thr-Glu-Thr-Asp-Phe-Arg

T3: Thr-Gly-Glu-Ala-Pro-Trp-Trp-Ser-Ala

c I) Thr-Leu-Ala-Thr-Phe-Gly-Ile-Asn-Ala-Val-Cys-Thr-His-Leu-Gly-Cys-Val-Val-Pro-Phe-Asn-Ala-Ala-Glu-Asn-Lys-Phe-Ile-Cys-Pro-Cys-His-Gly-(Ser-Trp-Tyr-Asn-Asn-Trp-...)

II) Gly-Pro-Ala-Pro-Leu-Ser-Leu-Ala-Leu-Ala-His-Cys-Asp-Val-Asp-Asp-Gly-Lys-Val-Val-Phe-Val-Pro-Trp-Thr-Glu-Thr-Asp-Phe-Arg

Fig.1. (a) N-terminal sequence of the FeS protein from spinach (96 residues). T1, T4, T5 and T6 are tryptic peptides which were isolated and sequenced. T4 shows that trypsin apparently retained some chymotryptic activity. (b) T1, T2 and T3 are tryptic peptides containing Trp. (c) Sequenced peptides containing cysteine from an endoproteinase Arg C digestion of the spinach Rieske protein. Cysteine residues were determined as *S*-(carboxymethyl)cysteine. Underlined peptides are conserved in the Rieske protein of *N. crassa* [5]. Parentheses indicate some uncertainty due to a minor contamination.

20 kDa protein electroeluted from the SDS gels that contained no iron was identical to the iron containing polypeptide purified according to Hurt et al. [8]. The initial yield in the gas-phase Edman degradation was 80–90% in both cases and there was no second sequence in either case. Therefore there was no indication of an impurity that might be blocked at the N-terminus.

For all further experiments the electroeluted protein was used. After CNBr cleavage of the FeS

protein at 16 kDa peptide was purified. The N-terminal sequence of this peptide started with residue 34, i.e. it overlapped the N-terminal sequence and continued to amino acid 77. Despite a large excess of CNBr (500-fold molar excess over methionine) we could not detect any further CNBr fragments using SDS-PAGE. From a tryptic digestion of the protein three tryptophan containing peptides were isolated by reverse-phase HPLC (fig.1b). The sequence of the peptide T1 confirmed the sequence of the CNBr fragment as it contained an overlapping sequence of residue 57–70. The tryptic peptide T3 is possibly the tentative carboxyl-terminal peptide of the FeS protein because it does not end with an arginine or lysine, but rather with an alanine. A combined cleavage at Met and Trp residues with CNBr yielded three peptides (4, 7 and 11 kDa according to SDS-PAGE). The N-terminal sequence of the 4 kDa peptide started at leucine 69 and its degradation was followed for another 28 steps (fig.1a). In this fragment exists an acid labile bond, aspartic acid 86-proline 87 which caused the appearance of the 11 kDa peptide. The 7 kDa band was a mixture of at least three different peptides as revealed by sequence analysis. The amino acid composition of the FeS protein is given in table 1. The combination of the partial sequences yielded a total sequence of 96 residues starting at the N-terminus, i.e. about half of the polypeptide. The partial N-terminal sequence from spinach Rieske FeS protein shows no significant homology to the sequences of mitochondrial FeS proteins which are already known from *N. crassa* [5] and *Rps. sphaeroides* [6]. Indeed major homologies are not expected at the N-terminus, but rather at the (2Fe-2S) cluster binding site. The cysteines which are assumed to bind the (2Fe-2S) cluster are not located in the first half of the protein. The sequence of two cysteine containing peptides was determined from an endoproteinase Arg C digestion of the protein prepared according to [17]. Either peptide contained a conserved box of four to eight amino acid residues (fig.1c) which is also present in the Rieske

Table 1

Amino acid composition of the Rieske iron-sulfur protein based on a molecular mass of 20 kDa

Amino acid	mol/mol	Nearest integer
Asp	13.2	13
Glu	10.7	11
Cys[a]	2.6	5[b]
Ser	7.9	8
Gly	19.7	20
Thr	14.0	14
Ala	20.6	21
His	3.4	3
Pro	16.6	17
Arg	5.8	6
Tyr	4.2	4
Val	15.4	15
Met	1.6	3[b]
Ile	7.3	7
Leu	21.7	22
Phe	8.4	8
Lys	6.8	7
Trp[c]	–	6
Total		190

[a] Determined as *S*-(carboxylmethyl)cysteine
[b] Estimated assuming 50% destruction during hydrolysis
[c] Determined from sequenced peptides

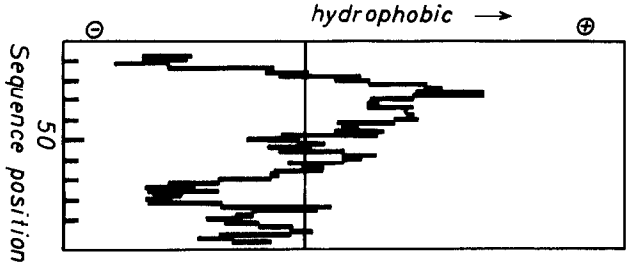


Fig.2. Hydropathy plot according to Kyte and Doolittle [18] of the 96 N-terminal amino acids from the 20 kDa protein of spinach. A window of 15 residues was taken for the computation.

iron-sulfur proteins from *Rps. sphaeroides* [6] and *N. crassa* [5], respectively. A conserved pentapeptide included a sequence Cys-Pro-Cys which seems characteristic for Rieske FeS proteins [5,6] and is involved in iron binding. The cysteine in the other peptide is not found in the conserved region and is therefore probably not involved in the binding of the FeS cluster.

The hydropathy plot analysis [18] for our partial sequence of the plant Rieske FeS center shows a long hydrophobic sequence from amino acid 20 to 56, i.e. longer than is necessary to span the membrane in a hydrophobic α-helix (fig.2). This is in agreement with the published mitochondrial sequences which also indicate a hydrophobic anchor to the membrane at the N-terminus.

ACKNOWLEDGEMENTS

The authors thank Mr H. Korte for skillful technical assistance. These investigations were supported by a grant of the Deutsche Forschungsgemeinschaft, SFB 168, to A. Trebst.

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