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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF THE HYDROPHOBIC ω SUBUNIT OF THE CHLOROPLAST ENERGY COUPLING COMPLEX

NORMAN E. TANDY*, RICHARD A. DILLEY and FRED E. REGNIER*
Department of Biological Sciences, Department of Biochemistry, Purdue University Biochemistry Program, Purdue University, W. Lafayette, IN 47907 (U.S.A.)

SUMMARY

The ω subunit of the coupling complex contains *ca.* 80% hydrophobic residues. It is therefore insoluble in aqueous buffers and can be most easily isolated in organic solvents. High-performance liquid chromatographic procedures were developed for rapid purification of the subunit by developing a Whatman M9 column of Partisil 10 ODS with step gradients of acetonitrile, methanol and trifluoroacetic acid. This procedure was limited by a smaller peptide, bound to and eluted with the ω subunit when the protein concentration on the column decreased. A preliminary purification by Sephadex LH-20 chromatography was necessary to remove lipids and/or other proteins, which, if not removed caused the column to become clogged.

An alternative procedure employed ion-exchange high-performance liquid chromatography on a Synchropak AX 300 column. The protein was applied in chloroform–methanol and eluted with a gradient of 3 m*M* to 20 m*M* ammonium acetate in chloroform–methanol–water. The latter procedure had the advantage of permitting chromatography of greater loads of impure protein solution without loss of resolution or clogging of the column.

INTRODUCTION

The ω subunit of the chloroplast coupling factor is a protein of the chloroplast coupling complex the function of which is to translocate protons through the membrane to the coupling factor on the membrane[1–3]. Some of the characteristics of this protein are that: (1) it is the site of binding of the energy-transfer inhibitor dicyclohexylcarbodiimide (DCCD)[2,3]; (2) it has a molecular weight of 8000; (3) it is extremely hydrophobic as shown in Table I[4]. A number of procedures have been reported for isolating the subunit, all of which employ extraction by organic solvents. The simplest procedure, reported by Nelson *et al.*[2], involves extraction of the chloroplasts with 1-butanol followed by ether precipitation to isolate the subunit. Un-

* Present address: Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, U.S.A.

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TABLE I

AMINO ACID COMPOSITION OF THE ω SUBUNIT

The amino acid composition in column 2 was determined from the sequence of the ω subunit published by Sebald and Wachter[4]. Column 3 is the composition of the peptide eluted from the reversed-phase column after 13 min. The amino acid composition was determined after hydrolysis for 24 h at 110°C with 6 *N* hydrochloric acid in sealed, evacuated tubes.

Amino acid	*From protein sequence*	*From reversed-phase HPLC*
Asx	2	2.6
Thr	3	3.2
Ser	3	3.2
Glx	7	5.3
Pro	4	4.3
Gly	11	10.9
Ala	17	15.8
Val	7	6.5
Met	2	1.7
Ile	6	5.8
Leu	12	12.4
Tyr	1	1.0
Phe	3	3.6
Lys	1	1.3
Arg	2	2.1

fortunately this yields protein with very limited solubility in anything except strong detergents[3], requires large volumes of ether, and gives low yields of protein. Other isolation procedures use either butanol[3] or chloroform–methanol extraction[5,6] followed by ion-exchange chromatography on either DEAE- or CM-cellulose[3,4]. Thin-layer chromatography[6] and reversed-phase high-performance liquid chromatography (HPLC)[7,8] have also been used.

This paper reports HPLC techniques for the purification of the ω subunit. Our reversed-phase system is unusual in that the sample is applied in organic solvent and the column is eluted by a gradient from acetonitrile to chloroform–methanol–trifluoroacetic acid. Although Hearn *et al.*[9] have reported that most polypeptides are retained on alkylsilane columns in high concentrations of organic solvent, this gradient protocol is the reverse of that normally used. The extreme hydrophobicity of this protein necessitated the use of an organic system. A limitation of the reversed-phase technique reported here for ω subunit is that resolution and recovery are dependent on sample load. An alternative HPLC procedure is presented that uses ion exchange but is unique in that organic solvents are used. The system is that described by Fillingame[5] for DEAE-cellulose chromatography.

MATERIALS AND METHODS

Chromatography

HPLC grade acetonitrile, chloroform, and methanol were obtained from American Scientific Products. Reversed-phase chromatography was carried out on Partisil 10 ODS columns (250 × 4.6 mm I.D. or 250 × 9.4 mm I.D.) obtained from

Whatman (Clifton, NJ, U.S.A.). The apparatus and elution procedure were as described previously[8].

High-performance ion-exchange chromatography (HPIEC) was performed with a microprocessor-controlled triple-pump system from Waters. One of the pumps was able to draw from any one of three reservoirs selected by a valve. The solvent system was essentially that described by Fillingame[5] for DEAE-cellulose chromatography. A Synchropak AX 300 (100 × 4.1 mm I.D.) column from SynChrom (Linden, IN, U.S.A.) was used and fractions of the eluent, coinciding with peaks of 280 nm absorbance, were collected. The collected eluent was desalted and concentrated as described previously[8].

Purification of the ω subunit

Chloroplasts were prepared by a modification of the procedure of Barr and Crane[10]. Washed, deveined leaf fragments were ground for 20 sec in 1 l of 0.4 *M* sucrose, 0.05 *M* sodium chloride. The homogenate was strained through twelve layers of cheesecloth, then a layer of Miracloth. The homogenate was centrifuged for 10 min at 2520 *g* and 0°C. The pellets were resuspended in 0.02 *M* sucrose, 5 m*M* N-(2-hydroxyethyl)-1-piperazine-N′-ethanesulfonic acid (HEPES) (pH 7.5), 2 m*M* magnesium chloride, and 0.5 mg/ml bovine serum albumin, then centrifuged for 10 min at 10,000 *g* and 0°C. The pellets were resuspended in 45 ml of 100 m*M* sucrose, 50 m*M* potassium chloride, 2 m*M* magnesium chloride, 50 m*M* HEPES adjusted to pH 8.6 with sodium hydroxide. The protein-to-chlorophyll ratio was estimated as described previously[11] to be 3.5:1. Protein extraction was performed as described previously[8]. After the fractions containing protein had been concentrated, the sample was chromatographed on an LH-20 column. Fractions containing the ω subunit were ether-precipitated, redissolved in 10 ml of chloroform–methanol (2:1) and subjected to HPLC.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

The gels were prepared as described previously[8]. The protein (1–2 mg/ml in 100 m*M* Tris-HCl (pH 8.6), 6 *M* urea, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.01% EDTA) was applied to the gels (12 × 13.5 × 0.2 cm; 15% acrylamide with 0.19% N,N′-methylene-bis-acrylamide in a buffer of 0.8 *M* Tris-HCl (pH 8.9), 2

TABLE II

PURIFICATION STEPS FOR THE ω SUBUNIT

Step	*Total protein yield (mg)*
Chloroplast membrane protein	1218*
Crude ether precipitate	44.1
LH-20 chromatography	44.3
Ether precipitate	35.6
Reversed-phase HPLC	5.12**
or	
Ion-exchange HPLC	8.15**

* Based 348 mg of chlorophyll, where the protein to chlorophyll ratio is 3.5 to 1[11].

** Reversed-phase and ion-exchange chromatography, followed by desalting and concentrating the collected eluates.

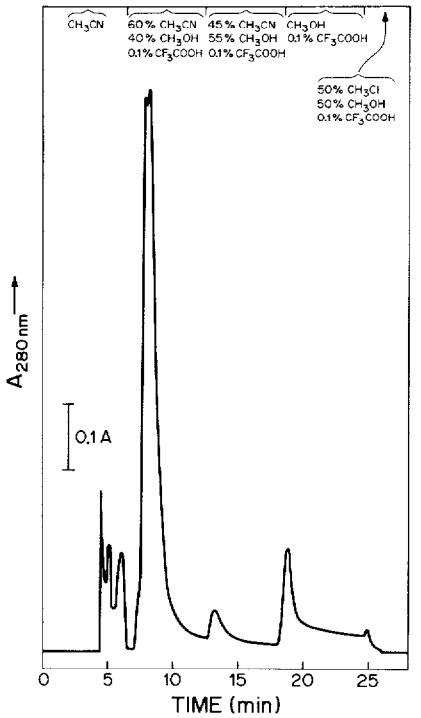

Fig. 1. Reversed-phase HPLC of the impure protein from the final ether precipitation. The impure protein (2.0 ml, 2.85 mg/ml) was applied to the Partisil 10 ODS column (25 × 0.94 cm I.D.) and eluted at 3 ml/min with the indicated step gradient. Absorbance of the eluate was monitored at 280 nm. The eluate containing the 13-min peak was collected.

m*M* EDTA, 0.1% SDS, 6 *M* urea). Polymerization was initiated by the addition of 0.06% N,N,N′,N′-tetramethylethylene diamine (TEMED) and 0.05% ammonium persulfate. The wells were formed in a stacking gel of 6% acrylamide, 0.094% N,N′-methylene-bis-acrylamide, 0.02 *M* Tris-HCl (pH 6.8), 0.1% SDS, 2 m*M* EDTA, 6 *M* urea, 0.05% ammonium persulfate 0.1% TEMED. Gels were electrophoresed at 12 mA/gel in a buffer of 0.025 *M* Tris-HCl (pH 8.6), 0.1% SDS, 0.2 *M* glycine, stained in 0.25% Comassie Brilliant Blue–25% 2-propanol–10% acetic acid at 40°C for 2 h, and destained for 4 h at 50°C in 10% 2-propanol–10% acetic acid at 50°C.

RESULTS

Table II summarizes the steps in the purification procedure and the total protein yield at each step. The procedure has been discussed in more detail in a previous paper[8]. Recovery of activity is not shown because the ω subunit has no measurable activity and the protein was not labeled with [^{14}C]DCCD as in earlier purifications[5]. Protein obtained by either the HPIEC or reversed-phase HPLC procedure was of comparable purity.

The elution protocol and chromatogram obtained from reversed-phase HPLC of the ω subunit on a Partisil 10 ODS column (25 × 0.94 cm I.D.) are shown in Fig. 1.

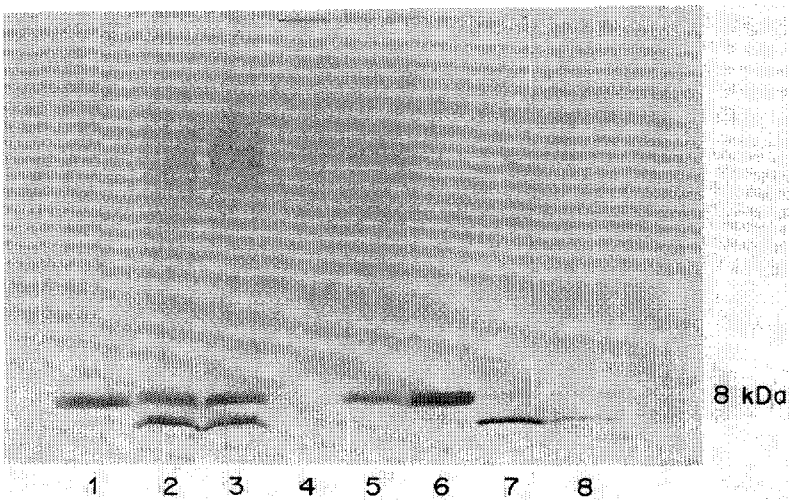

Fig. 2. SDS-polyacrylamide gel electrophoresis of the collected peaks from HPLC. Lane 1 contained 20 μg of protein collected from the reversed-phase HPLC column (25 × 0.94 cm I.D.) (Fig. 1) 13 min after application of 2 ml of 2.85 mg/ml impure protein to the column. Lane 2 contained 20 μl of a solution of all of the protein collected from the reversed-phase HPLC column 13 min after application of 0.2 ml of 3 mg/ml impure protein to the column. Lane 3 contained 20 μl of a solution of all the protein collected from the reversed-phase column 13 min HPLC after application of 2 ml of 0.3 mg/ml impure protein to the column. Lane 4 contained the protein from attempts to chromatograph the impure protein from the final ether precipitation directly on the 25 × 0.46 cm I.D. Partisil 10 ODS column. Lane 5 contained 20 μl of solution obtained by applying 2 ml of 0.6 mg/ml impure protein to the reversed-phase HPLC column (25 × 0.94 cm I.D.), collecting the 13-min peak, desalting and concentrating to 0.5 ml, then applying the residue to the analytical Partisil column (25 × 0.46 cm I.D.). The 13-min peak material from the analytical column was collected, desalted, dried and redissolved in 20 μl SDS-urea buffer. Lane 6 contained 30 μg of protein collected from the ion exchange column (see Fig. 4) 38 min after application of 2 ml of 2.85 mg/ml impure protein to the column. Lane 7 contained 30 μg of protein collected from the ion-exchange column after 43 min. Lane 8 contained 30 μg of protein collected from the ion-exchange column after 47 min. kDa = kilodaltons.

Amino acid composition (Table I), sequence analysis, and DCCD labeling have shown the protein eluted after 13 min to be the ω subunit[8]. The protein yield by reversed-phase HPLC reported in Table II is from this 13-min peak material. The subunit is essentially pure as shown by lane 1 in Fig. 2 and by previous work[8]. However, the purity after reversed-phase HPLC was found to be dependent on the amount of protein applied to the column. As shown in Fig. 2, when 2 ml of protein solution at 2.85 mg/ml was applied to the column, the protein in the 13-min peak was nearly pure (lane 1). In contrast, when 0.2 ml of a 3.0 mg/ml solution (lane 2) or 2 ml of a 0.3 mg/ml solution (lane 3) were applied to the same column, the 13-min peak material was a mixture of peptides.

Fig. 3 shows more clearly the dependence of purity on the sample load. For each lane, 1 ml of solution containing the number of milligrams of protein indicated beneath each lane was applied to the column and chromatographed as described in Fig. 1. These chromatograms are shown in Fig. 4. Eluate coinciding with the peak at 13 min was collected, dried, and redissolved in the buffer for electrophoresis. Increasing volumes of the solution (from 10 to 60 μl) were applied to the gel lanes in an

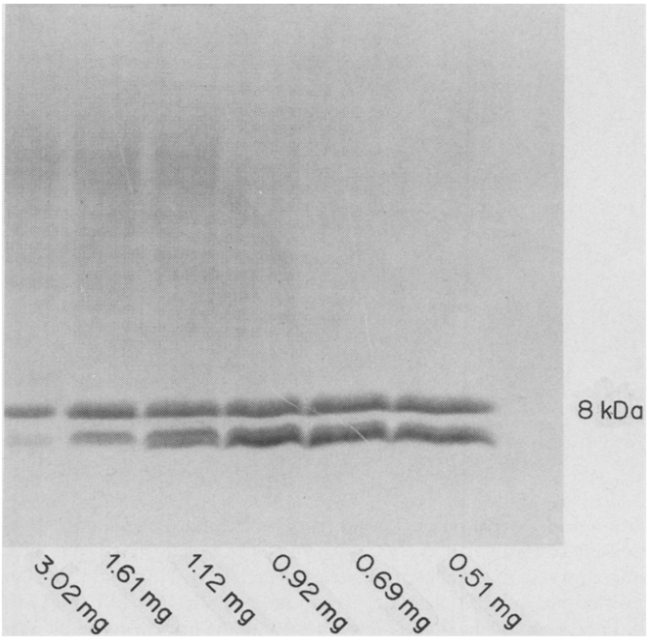

Fig. 3. SDS-polyacrylamide gel electrophoresis of the 13-min peaks from the reversed-phase HPLC column (25 × 0.94 cm I.D.) as a function of protein load. The gel was prepared as described in Methods. The numbers below each lane indicate the milligrams of impure protein applied to the column in 1 ml of chloroform–methanol (2:1) solution.

attempt to keep the protein load on the gel nearly constant. It is seen that, as more protein was applied to the Partisil 10 ODS column, the purity of the protein in the 13-min peak increased. The change in the relative area of the peaks in Fig. 4 suggests that at lower sample loads, the peptides in the 8-min peak might be bound to the peptide in the 13-min peak and eluted together. While the relative areas of the other peaks remained nearly constant, the 8-min peak changed from much larger than the 13-min peak to smaller than the 13-min peak (see also Fig. 1).

An attempt was made to chromatograph the impure protein directly on an analytical Partisil 10 ODS column (25 × 0.46 cm I.D.) (lane 4, Fig. 2). The problem with this approach was that even very small protein loads would cause column pressure to exceed 4500 p.s.i. when the pump was operated at a flow-rate greater than 0.5 ml/min. This happened with a protein load of 0.18 mg, which would have yielded only *ca.* 25 μg of ω subunit. An alternative procedure was to load small amounts of protein on to the large column and to elute the protein as in Fig. 1, collecting the 13-min peak. This material could then be concentrated, desalted, and chromatographed on the analytical column at 0.7–1.0 ml/min as described in Fig. 1, but with 4-min gradient steps. The yield from this procedure is shown on lane 5 of Fig. 2. Rechromatographing the 13-min peak material on the 25 × 0.94 cm I.D. column did not purity (data not shown).

An alternative to reversed-phase HPLC is HPIEC, as shown in Fig. 5. The elution scheme was essentially that described by Fillingame[5] for DEAE-cellulose, but

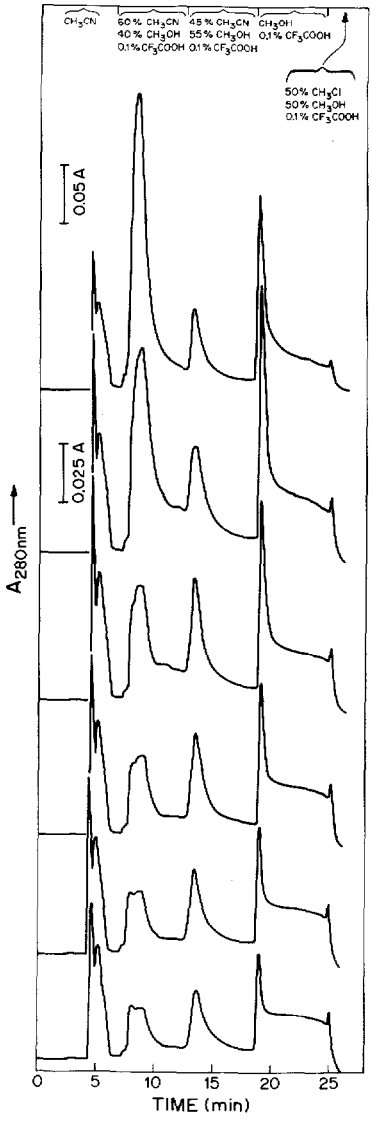

Fig. 4. Reversed-phase HPLC of decreasing concentrations of impure protein from the isolation of the ω subunit. Solutions (1 ml) with various concentrations of protein were applied to the Whatman Partisil 10 ODS column (25 × 0.94 cm I.D.) and eluted at 3 ml/min with the gradient indicated. Absorbance was monitored at 280 nm. The calibration bar at the top is for the top chromatogram. The second calibration bar is for the rest of the chromatograms. Amounts of protein loaded were (from top to bottom): 3.02 mg, 1.61 mg, 1.12 mg, 0.92 mg, 0.69 mg, 0.51 mg.

a Synchropak AX 300 column (10 × 0.41 cm I.D.) was used. The ω subunit isolated by this procedure was essentially as pure as that obtained with the reversed-phase column (Fig. 2, lanes 1 and 6). The ion-exchange column did not develop the back-pressure problems encountered with the reversed-phase column, even though the same load (2 ml of 2.85 m/ml) was placed on a much smaller column. A 10 × 0.41 I.D.

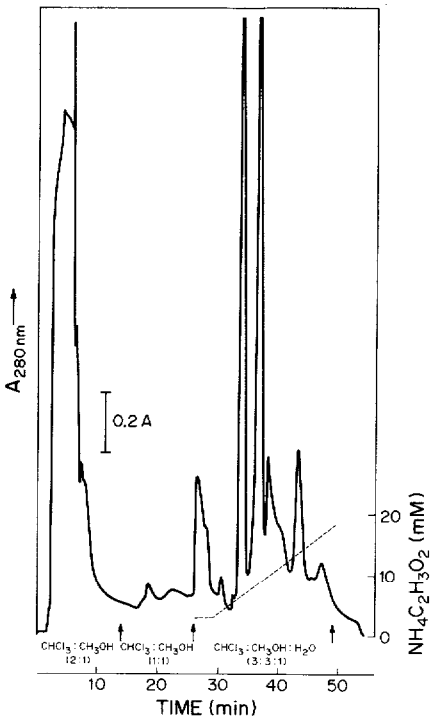

Fig. 5. HPIEC of the impure protein from the final ether precipitation. Impure protein (2.0 ml, 2.85 mg/ml) was applied to the Synchropak AX 300 column and eluted at 0.5 ml/min with the gradient indicated in the figure as adapted from Fillingame[5]. Absorbance of the eluate was monitored at 280 nm. Peak fractions at 38, 43 and 47 min were collected.

HPIEC column was used, compared with a 25 × 0.94 cm I.D. reversed-phase column. The pressure on the HPIEC column never exceeded 300 p.s.i. whereas the pressure in reversed-phase HPLC exceeded 3000 p.s.i. before the non-retained peak was eluted. Also no upper or lower load limit for the HPIEC column has yet been found in these studies. Both 1.4 and 5.6 mg of the crude protein gave the same chromatogram on the AX 300 column (data not shown).

DISCUSSION

The elution protocol used for the reversed-phase HPLC column is unusual in that it is the reverse of that normally reported[12]. Although it should be noted that Mahoney and Hermodson[13] have experimentally determined a reversed-phase HPLC eluotropic series that follows neither the solvent strength parameter nor the polarity parameter, such gradients usually proceed from a solvent with higher solvent strength parameter[14] to one of lower strength. It has been observed that polypeptides are sorbed on alkylsilane columns from two kinds of eluent: (1) aqueous buffers and (2) high concentrations of organic solvent[9]. Strongest retention was found at the extremes of solvent polarity, while elution was achieved by intermediate concentrations of organic solvent. Observations by Hearn *et al.*[9] suggested that gradient elution from

high organic solvent concentration to increasing amounts of water should also be possible. Our studies confirm that in the case of the ω subunit, this later mode of elution is in fact the most useful.

A serious problem of the reversed-phase HPLC system is the loss of purity in the 13-min peak material as the protein load on the column decreases. When the chromatograms for Fig. 3 were prepared, the 8-min peak was observed to decrease faster than the 13-min peak as the load decreased, until the two peaks were of equal size. The primary component of the 8-min peak is of the same molecular weight as the primary contaminant of the 13-min peak (unpublished data). Thus a possible explanation for the increasing contamination is that as the peptide in the 8-min peak becomes more dilute and hydrophobic interactions with itself decrease, it may be bound to the ω subunit, which is still bound to the column, and be eluted together with the ω subunit in the 13-min peak.

In summary, either reversed-phase or ion-exchange HPLC can be used to purify the ω subunit. Ion-exchange chromatography would obviously be the procedure of choice because it has broader load limits, operates at lower pressure and requires less expensive columns. DCCD, which covalently modifies a glutamate residue of the ω subunit[4], has been shown to change the charge on the subunit sufficiently to change its retention time on the ion-exchange column[5]. This change has not been seen on the reversed-phase column[8]. However, this change can also be used to separate subunits after reaction with different reagents[15]. Other organic solvent systems used with DEAE-cellulose for the fractionation of lipids and other proteins[16–18] could probably also be used with the Synchropak AX 300 column.

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