# Subunit *a* of the *E. coli* ATP synthase: reconstitution and high resolution NMR with protein purified in a mixed polarity solvent

Oleg Y. Dmitriev[a], Karlheinz Altendorf[b], Robert H. Fillingame[a,*]

[a] *Department of Biomolecular Chemistry, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706-1532, USA*
[b] *Fachbereich Biologie/Chemie, Universität Osnabrück, Barbarastr. 11, 49069 Osnabrück, Germany*

Received 4 November 2003; accepted 5 November 2003

First published online 26 November 2003

Edited by Richard Cogdell

**Abstract Subunit *a* of the *Escherichia coli* ATP synthase, a 30 kDa integral membrane protein, was purified to homogeneity by a novel procedure incorporating selective extraction into a monophasic mixture of chloroform, methanol and water, followed by Ni-NTA chromatography in the mixed solvent. Pure subunit *a* was reconstituted with subunits *b* and *c* and phospholipids to form a functional proton-translocating unit. Nuclear magnetic resonance (NMR) spectra of the pure subunit *a* in the mixed solvent show good chemical shift dispersion and demonstrate the potential of the solvent mixture for NMR studies of the large membrane proteins that are currently intractable in aqueous detergent solutions.**


*Key words:* ATP synthase; Subunit *a*; Membrane protein purification; Chloroform–methanol–water solvent; Proton translocation; TROSY

## 1. Introduction

Integral membrane proteins present significant experimental difficulties in purification and structural studies. Purification of membrane proteins in aqueous detergent solutions requires extensive screening for solubilization, retention of activity and adaptation of protein fractionation methods like Ni-NTA chromatography. High-resolution nuclear magnetic resonance (NMR) of membrane proteins purified in aqueous detergent solutions presents especially difficult challenges. A molecule of membrane protein associated with several dozens detergent molecules in the micelle has significantly longer correlation time than a molecule of water-soluble protein of comparable size. Longer correlation time translates into fast relaxation of NMR signals, which in turn leads to poor resolution and loss of spectral information. For this reason, with the exception of small peptides, the only integral membrane protein structures solved to date by NMR in aqueous detergent solution are two medium-sized β-barrel proteins [1,2].

Integral membrane proteins can be extracted into mixtures of organic solvents and water, an approach originally developed for extracting lipids. Examples of proteins successfully extracted and purified in a mixture of chloroform, methanol and water include subunit *c* of the ATP synthase [3] and a small multidrug transporter EmrE from *Escherichia coli*. Following purification in a chloroform–methanol–water mixture, subunit *c* was reconstituted with phospholipids and detergent-purified subunits *a* and *b*, and proton channel function was demonstrated [4]. The EmrE transporter was also shown to retain function after purification in a chloroform–methanol–water mixture [5].

The structure of the ATP synthase subunit *c* in chloroform–methanol–water was solved by NMR [6]. This structure agreed well with biochemical and genetic data for the protein in situ and is consistent with moderate resolution electron density maps of subunit *c* oligomers from bovine mitochondria [7] and *Ilyobacter tartaricus* [8]. The structures of two mutant forms of subunit *c* [9,10] and of the membrane domain of ATP synthase subunit *b* [11] have also been solved by NMR in this mixed polarity solvent. The NMR investigation of EmrE in a similar chloroform–methanol–water mixture produced chemical shift assignments for many of the backbone and side chain resonances and secondary structure predictions but did not yield a three-dimensional structure [12]. Further information is needed to validate the use of such solvent mixtures for structural studies of membrane proteins.

We are currently investigating the structure of transmembrane subunit *a* of the *E. coli* ATP synthase. $H^+$-transporting $F_1F_0$ ATP synthases utilize the energy of an $H^+$ electrochemical gradient to drive formation of ATP from ADP and $P_i$. The enzymes are composed of distinct extramembranous and transmembranous sectors, termed $F_1$ and $F_0$, respectively. Proton movement through $F_0$ is reversibly coupled to ATP synthesis or hydrolysis in catalytic sites on $F_1$. Each sector of the enzyme is composed of multiple subunits with the simplest composition being $\alpha_3\beta_3\gamma\delta\varepsilon$ for $F_1$ and $a_1b_2c_{10}$ for $F_0$ in the case of the *E. coli* enzyme [13–15]. The structure of the $F_1$ complex has been solved by X-ray crystallography [16,17], but the efforts to crystallize complete ATP synthase or the transmembrane $F_0$ complex so far have not met with success. The proton pathway through $F_0$ is believed to lie at the interface of an oligomeric ring of subunit *c* and subunit *a* [18]. The structure of subunit *a* is therefore of particular importance in understanding the molecular mechanism of ion translocation through $F_0$. Subunit *a* is 271 amino acids long and is predicted to fold with five transmembrane helices [19–21]. Helix IV is believed to be in direct contact with the outer surface of the cylindrical subunit *c* oligomer [22,23].

We have devised a method for purification of subunit *a* in a chloroform–methanol–water solvent mixture that may be generally applicable to the purification of other membrane pro-

*Corresponding author. Fax: (1)-608-262 5253.
*E-mail addresses:* altendorf@biologie.uni-osnabrueck.de (K. Altendorf), rhfillin@facstaff.wisc.edu (R.H. Fillingame).

0014-5793/03/$30.00 
doi:10.1016/S0014-5793(03)01360-7

teins. Passage through the solvent mixture does not perturb biological function of the protein following reconstitution into phospholipid liposomes. Well-resolved NMR spectra of the purified protein have been obtained using TROSY methods [24].

## 2. Materials and methods

### 2.1. Bacterial strain and cultivation

Subunit *a* was purified from strain OM202 transformed with plasmid pBWU13 [25]. Strain OM202 has a deletion of the complete *atp* operon. The pBWU13 plasmid is derived from pBR322 and contains the entire *atp* operon. The *atpB* gene encoding subunit *a* was modified to include a hexahistidine tag immediately after the N-terminal methionine. The starter culture was grown on LB broth and inoculated at a ratio of 1:100 (v/v) into a minimal medium containing 0.1 M potassium phosphate, pH 7.0, 15 mM $NH_4Cl$, 15 mM $Na_2SO_4$, 1.8 μM $FeSO_4$, supplemented with 3 g/l glucose, 2 mg/l thiamine, and 400 mg/l ampicillin. The bacterial culture was grown aerobically to late exponential phase and cells were collected by centrifugation.

### 2.2. Extraction of subunit a from the membranes

Cells were suspended and homogenized in 150 mM $CH_3COONa$, pH 4.0, added at a ratio of 0.5 ml/g wet weight. The cell suspension was added to 6.5 volumes of a stirring mixture $CHCl_3$:$CH_3OH$ (1:1, v/v) and the pH adjusted to 4.5. The mixture was stirred for 2 h at 4°C. Cell debris was removed by centrifugation in glass bottles at $1000\times g$ for 15 min. Chloroform and water were added to the clear supernatant in the amount of 0.43 parts and 0.18 parts by volume respectively. After gentle mixing, the phases were allowed to separate overnight in a separatory funnel. An equal volume of chloroform was added to the lower phase with dropwise addition of methanol when necessary to keep the solution clear, and then the solution was dried by rotor evaporation at 37°C. The dry residue, consisting of lipid and membrane protein, was redissolved in $CHCl_3$:$CH_3OH$ (2:1) at a ratio of 0.4 ml/g starting cell mass. This solution was added to five parts chilled diethyl ether (v/v) and incubated at −20°C overnight. The precipitated protein was collected by centrifugation at $1000\times g$ for 20 min and redissolved in $CHCl_3$:$CH_3OH$:$H_2O$ (4:4:1) containing 5 mM $CH_3COONa$, pH 4.5 at a ratio of 0.5 ml/g initial cell mass. Subunit *a* was purified from this solution either by acetone fractionation and ion exchange chromatography for the wild type protein, or by Ni-NTA chromatography for the His-tagged protein.

### 2.3. Purification of the wild type (no His tag) subunit a

The pH of the protein solution was adjusted to 6.0 and the precipitated protein removed by centrifugation. The supernatant was mixed with 0.25 parts by volume of chilled acetone and incubated for 2 h at −20°C. The protein precipitate was redissolved in $CHCl_3$:$CH_3OH$:$H_2O$ (4:4:1) containing 5 mM $CH_3COONa$, 1 mM LiCl, pH 6.0 and applied to a CM-cellulose column preequilibrated with the same buffer. After washing the column with the equilibration buffer, subunit *a* was eluted with a linear concentration gradient of 1–20 mM LiCl in the equilibration buffer and concentrated by acetone precipitation. Protein purity was assessed by sodium dodecyl sulfate (SDS) electrophoresis [26] and silver staining [27]. The sample was prepared by addition of 1 volume of protein in chloroform–methanol–water (4:4:1) solvent to 9 volumes of SDS sample buffer, followed by heating at 70°C for 15 min.

### 2.4. Purification of His-tagged subunit a by Ni-NTA chromatography

A chromatography column was filled with Ni-NTA agarose (Qiagen), equilibrated in $CHCl_3$:$CH_3OH$:$H_2O$ (4:4:1) containing 5 mM $CH_3COONa$, pH 4.5, in the amount of 0.15 ml packed volume per gram starting biomass. After the protein solution was applied to the column, it was washed with five bed volumes of $CHCl_3$:$CH_3OH$:$H_2O$ (4:4:1) containing 50 mM imidazole and 5 mM $CH_3COONa$, pH 4.5. Protein was eluted with $CHCl_3$:$CH_3OH$:$H_2O$ (4:4:1) containing 0.1 M imidazole, 20 mM 1,10-phenanthroline and 5 mM $CH_3COONa$, pH 6.0. The protein was precipitated by adding 9 volumes chilled acetone at 0°C and collected by centrifugation at $1000\times g$ for 15 min. The precipitate was redissolved in $CHCl_3$:$CH_3OH$:$H_2O$ (4:4:1) containing 25 mM $CH_3COONa$, pH 5.5. Protein purity was assessed as described above.

### 2.5. Reconstitution in proteoliposomes and protein translocation assay

Subunits and complete $F_0$ were purified and reconstituted essentially as described previously [4]. For each 10 mg of soybean phospholipid the following amounts of $F_0$ or purified subunits were added: 125 μg complete $F_0$, 25 μg subunit *a*, 29 μg subunit *b*, 70 μg subunit *c*.

### 2.6. NMR spectroscopy

For NMR, bacterial cells were grown as described above, except that $^{15}NH_4Cl$ of 98% isotopic purity (Cambridge Isotope Laboratories) was substituted for the natural abundance $NH_4Cl$, and 90% $D_2O$ was used instead of $H_2O$ for preparing growth medium. The sample contained 0.3 mM subunit *a* in $CDCl_3$:$CD_3OH$:$H_2O$ (4:4:1), containing 25 mM $CD_3COONa$, pH 5.5. The [$^{15}N$,$^{1}H$]-TROSY NMR spectrum [24] was recorded at 27°C on a Bruker DMX-750 spectrometer. The spectrum was acquired with 200 complex points in $t_1$, 16 transients per point, and processed using Felix-2000 software (Accelrys).

## 3. Results and discussion

### 3.1. Purification

Subunit *a* of the ATP synthase was purified in a chloroform–methanol–water solvent mixture that was previously used for purification and structural studies of the subunit *c* of the ATP synthase. Preliminary experiments demonstrated that the composition of membrane proteins extracted into the chloroform–methanol–water varies with pH of the solvent. This allows adjusting the selectivity of extraction. For subunit *a*, the highest yield was observed at pH < 5.0, while more selective extraction was observed at pH 6.0. Generally, we found that progressively more proteins were found in the extract as pH was lowered from 7.0 to 4.0. Protein extraction was followed by removal of the insoluble material, phase separation and delipidation. These steps are essentially similar to those used for subunit *c* purification [3]. In the preliminary experiments, wild type subunit *a*, without the polyhistidine tag, was purified by acetone fractionation followed by ion exchange chromatography. The identity of the protein was confirmed by N-terminal peptide sequencing.

Purification of His-tagged subunit *a* on Ni-NTA agarose proved to yield protein of higher purity than the acetone fractionation/ion exchange procedure used for wild type subunit *a*, a decisive advantage for preparing samples for NMR. Binding of the hexahistidine-tagged subunit *a* to Ni-NTA agarose proved to be exceptionally tight. This is consistent with stronger electrostatic interactions in low-dielectric solvent compared to water. In aqueous solvent, protein purification procedures with Ni-NTA agarose are usually optimized by adjusting imidazole concentrations in elution buffers so that contaminating proteins are removed during column washing steps and the polyhistidine-tagged protein released at a higher imidazole concentration in the final elution buffer. Subunit *a* in chloroform–methanol–water solvent was not released from Ni-NTA even at high imidazole concentration. Protein elution was achieved using 1,10-phenanthroline, a metal chelator that is soluble in chloroform–methanol–water solvent. Subunit *a* purified by this procedure was essentially homogeneous (Fig. 1). The yield of purified protein was 0.12–0.15 mg/g wet weight of cells. The successful application of Ni-NTA resin chromatography in this organic solvent–water mixture should make this purification method adaptable to other integral membrane proteins.

### 3.2. Reconstitution

Wild type subunit *a* purified by acetone fractionation and

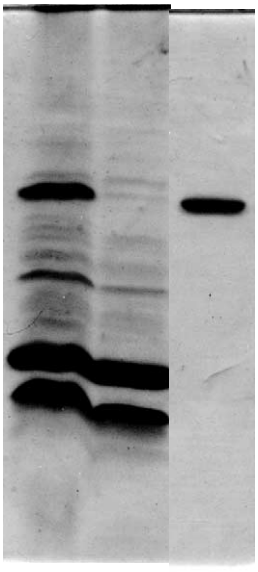



Fig. 1. Silver-stained SDS–polyacrylamide gel showing crude the cell extract (1), the Ni-NTA column flow-through impurities (2), and the final Ni-NTA elution containing purified subunit *a* (3).

ion exchange chromatography was reconstituted with subunits *b* and *c* of the *E. coli* ATP synthase and phospholipids in proteoliposomes. Imposition of a transmembrane $K^+$ electrochemical gradient induced rapid proton uptake in these proteoliposomes (Fig. 2). The formation of functional proton channels using subunit *a* after purification in chloroform–methanol–water demonstrated that the protein was not irreversibly denatured in this solvent. Reconstitution of activity had previously been demonstrated for subunit *c* and EmrE after purification in chloroform–methanol–water mixtures [4,5].

### 3.3. NMR spectroscopy

A $^1H$,$^{15}N$ chemical shift correlation NMR spectrum was recorded to assess the state of isolated subunit *a* in chloroform–methanol–water solvent and its suitability for NMR structural studies (Fig. 3). The fingerprint spectrum of subunit *a* shows good dispersion of chemical shifts, a characteristic expected of a folded protein. The range of backbone amide chemical shifts is consistent with large segments of α-helical secondary structure. The number of signals from easily identifiable residue types, e.g. Gly and the side chains of Trp and Arg, is consistent with the number of these residues in subunit *a*. For example, six of the seven Trp indoles in the protein are well resolved from each other.

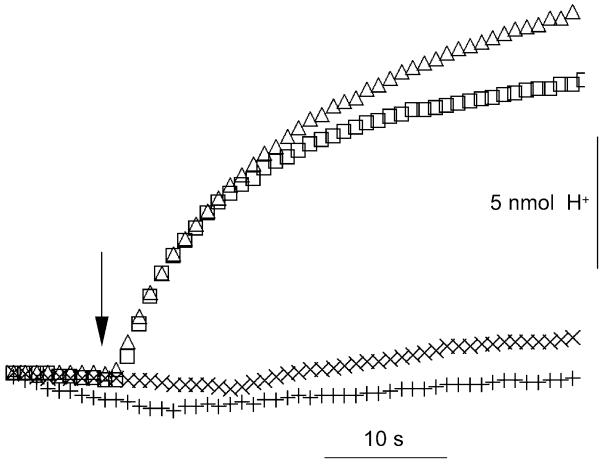
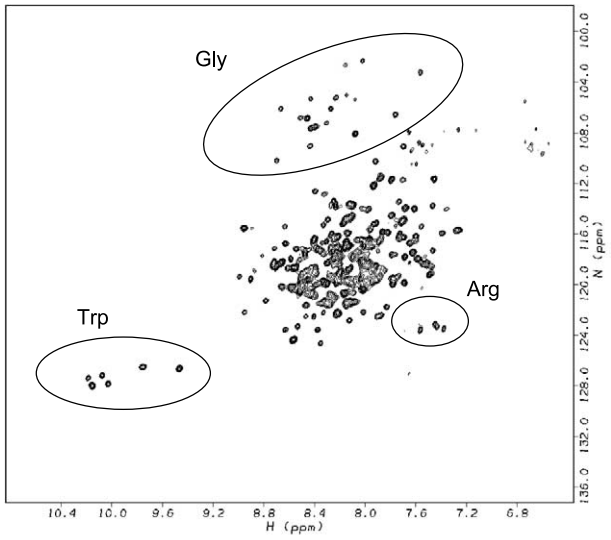



Fig. 2. Proton uptake in the proteoliposomes containing subunit *a* purified in chloroform–methanol–water mixture after reconstitution with subunits *b* and *c*. Proteoliposomes with subunit *c* only (×), subunits *a* and *b* only (+), subunits *a*, *b* and *c* (△), or purified complete $F_0$ complex (□).




MASENMTPQD YIGHHLNNLQ LDLRTFSLVD PQNPPATFWT INIDSMFFSV
VLGLLFLVLF RSVAKKATSG VPGKFQTAIE LVIGFVNGSV KDMYHGKSKL
IAPLALTIFV WVFLMNLMDL LPIDLLPYIA EHVLGLPALR VVPSADVNVT
LSMALGVFIL ILFYSIKMKG IGGFTKELTL QPFNHWAFIP VNLILEGVSL
LSKPVSLGLR LFGNMYAGEL IFILIAGLLP WWSQWILNVP WAIFHILIIT
LQAFIFMVLT IVYLSMASEE H

Fig. 3. A 750 MHz [$^{15}N$,$^1H$]-TROSY spectrum of the purified His-tagged subunit *a* in chloroform–methanol–water. Signals from easily identifiable residue types are circled. The amino acid sequence of subunit *a* is shown in the lower part of the figure. Transmembrane α-helices, as predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), are shown in bold lettering.

A nearly complete chemical shift assignment of subunit *a* required for high-resolution NMR structure determination by NOE analysis is likely to be very difficult because of the size of the protein, the predominantly α-helical secondary structure and pronounced chemical shift degeneracy. An efficient strategy for structure determination will have to rely on more readily achieved backbone chemical shift assignments, and selective isotope labeling schemes to simplify NOE analysis. Our immediate goal is to identify α-helical segments in the subunit *a* structure and determine their folding in three-dimensional space.

## 4. Conclusions

We present a procedure for extracting and purifying subunit *a* in an organic solvent of mixed polarity. This method is simple and yields protein of high purity, and may easily be adapted to other integral membrane proteins. As demonstrated by functional reconstitution of the purified subunit *a*, passage through organic solvent–water mixture does not cause irreversible protein denaturation. The fingerprint NMR spectrum of the pure subunit *a* in chloroform–methanol–water

indicates a well-structured protein suitable for further investigation by NMR.

*Acknowledgements:* We thank Laura Oesterle for dedicated technical assistance. This research was supported by US Public Health Service Grant GM-23105, the Deutsche Forschungsgemeinschaft (SFB 431), and by an Alexander von Humboldt fellowship to O.Y.D. NMR studies were carried out at the National Magnetic Resonance Facility at Madison with support from the NIH Biomedical Technology Program (RR02301) and additional equipment funding from the University of Wisconsin, NSF Academic Infrastructure Program (BIR-9214394), NIH Shared Instrumentation Program (RR02781, RR08438), NIH Research Collaborations to Provide 900 MHz NMR Spectroscopy (GM66326), NSF Biological Instrumentation Program (DMB-8415048), and U.S. Department of Agriculture.

## References

[1] Arora, A., Abildgaard, F., Bushweller, J.H. and Tamm, L.K. (2001) Nat. Struct. Biol. 8, 334–338.
[2] Fernandez, C., Hilty, C., Wider, G. and Wuthrich, K. (2002) Proc. Natl. Acad. Sci. USA 99, 13533–13537.
[3] Fillingame, R.H. (1976) J. Biol. Chem. 251, 6630–6637.
[4] Dmitriev, O.Y., Altendorf, K. and Fillingame, R.H. (1995) Eur. J. Biochem. 233, 478–483.
[5] Yerushalmi, H., Lebendiker, M. and Schuldiner, S. (1995) J. Biol. Chem. 270, 6856–6863.
[6] Girvin, M.E., Rastogi, V.K., Abildgaard, F., Markley, J.L. and Fillingame, R.H. (1998) Biochemistry 37, 8817–8824.
[7] Stock, D., Leslie, A.G. and Walker, J.E. (1999) Science 286, 1700–1705.
[8] Vonck, J., von Nidda, T.K., Meier, T., Matthey, U., Mills, D.J., Kuhlbrandt, W. and Dimroth, P. (2002) J. Mol. Biol. 321, 307–316.
[9] Dmitriev, O.Y. and Fillingame, R.H. (2001) J. Biol. Chem. 276, 27449–27454.
[10] Dmitriev, O.Y., Abildgaard, F., Markley, J.L. and Fillingame, R.H. (2002) Biochemistry 41, 5537–5547.
[11] Dmitriev, O., Jones, P.C., Jiang, W. and Fillingame, R.H. (1999) J. Biol. Chem. 274, 15598–15604.
[12] Schwaiger, M., Lebendiker, M., Yerushalmi, H., Coles, M., Groger, A., Schwarz, C., Schuldiner, S. and Kessler, H. (1998) Eur. J. Biochem. 254, 610–619.
[13] Foster, D.L. and Fillingame, R.H. (1982) J. Biol. Chem. 257, 2009–2015.
[14] Jiang, W., Hermolin, J. and Fillingame, R.H. (2001) Proc. Natl. Acad. Sci. USA 98, 4966–4971.
[15] Fillingame, R.H., Jiang, W. and Dmitriev, O.Y. (2000) J. Exp. Biol. 203, 9–17.
[16] Abrahams, J.P., Leslie, A.G., Lutter, R. and Walker, J.E. (1994) Nature 370, 621–628.
[17] Gibbons, C., Montgomery, M.G., Leslie, A.G. and Walker, J.E. (2000) Nat. Struct. Biol. 7, 1055–1061.
[18] Fillingame, R.H. and Dmitriev, O.Y. (2002) Biochim. Biophys. Acta 1565, 232–245.
[19] Valiyaveetil, F.I. and Fillingame, R.H. (1998) J. Biol. Chem. 273, 16241–16247.
[20] Wada, T., Long, J.C., Zhang, D. and Vik, S.B. (1999) J. Biol. Chem. 274, 17353–17357.
[21] Zhang, D. and Vik, S.B. (2003) Biochemistry 42, 331–337.
[22] Jiang, W. and Fillingame, R.H. (1998) Proc. Natl. Acad. Sci. USA 95, 6607–6612.
[23] Angevine, C.M. and Fillingame, R.H. (2003) J. Biol. Chem. 278, 6066–6074.
[24] Pervushin, K., Riek, R., Wider, G. and Wuthrich, K. (1997) Proc. Natl. Acad. Sci. USA 94, 12366–12371.
[25] Iwamoto, A., Omote, H., Hanada, H., Tomioka, N., Itai, A., Maeda, M. and Futai, M. (1991) J. Biol. Chem. 266, 16350–16355.
[26] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 366–379.
[27] Heukeshoven, J. and Dernick, R. (1988) Electrophoresis 9, 28–32.