Structure and Function of Subunit *a* of the ATP Synthase of *Escherichia coli*

Steven B. Vik[1,2] and Robert R. Ishmukhametov[1]

The structure of subunit *a* of the *Escherichia coli* ATP synthase has been probed by construction of more than one hundred monocysteine substitutions. Surface labeling with 3-*N*-maleimidyl-propionyl biocytin (MPB) has defined five transmembrane helices, the orientation of the protein in the membrane, and information about the relative exposure of the loops connecting these helices. Cross-linking studies using TFPAM-3 (*N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimido-propionamide) and benzophenone-4-maleimide have revealed which elements of subunit *a* are near subunits *b* and *c*. Use of a chemical protease reagent, 5-(-bromoacetamido)-1,10-phenanthroline-copper, has indicated that the periplasmic end of transmembrane helix 5 is near that of transmembrane helix 2.

KEY WORDS: ATP synthase; subunit *a*; proton translocation; cysteine; mutagenesis; membrane topology.

INTRODUCTION

The ATP synthase from *E. coli* is an F-type ATPase that both synthesizes ATP in response to an electrochemical proton gradient, and transports protons in response to ATP hydrolysis (for a review see Capaldi and Aggeler, 2002). This enzyme is composed of eight different types of subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10}$ (Bragg and Hou, 1975; Foster and Fillingame, 1982). The three catalytic sites of the enzyme are formed by the $\alpha_3\beta_3$ hexamer. The peripheral stalk, δ and b_2, connects the $\alpha_3\beta_3$ hexamer to subunit *a*, forming the stator. The central stalk, consisting of $\gamma\varepsilon\,c_{10}$, rotates relative to the catalytic sites during enzyme turnover. Only two subunits, monomeric *a* and oligomeric *c*, appear to be directly involved in proton translocation across the membrane.

The key functional residue of subunit *a* is Arg 210 (Cain and Simoni, 1989; Lightowlers *et al.*, 1987), which appears to function through its interactions with c subunits, rather than undergoing protonation and deprotonation. The key functional residue of subunits *c* is Asp 61, which is the primary proton carrier. Other important residues of subunit *a* form half-channels from the cytoplasmic and the periplasmic surfaces to the center of the membrane where Asp 61 resides (Angevine and Fillingame, 2003; Angevine *et al.*, 2003). The following model has been proposed (Vik and Antonio, 1994; Vik *et al.*, 1998): At the interface of *a* and *c* subunits, two Asp 61 are deprotonated, one of which interacts with Arg 210. The one that interacts with Arg 210 can be protonated via an access channel through subunit *a* that is connected to the periplasm. When this occurs, rotation is driven in the direction which allows the neutralized Asp 61 to enter the lipid phase, and the next Asp 61 to move towards Arg 210. Translocated protons are released to the cytoplasm as *c* subunits enter the interface with subunit *a*, through a second access channel (see Fig. 1). Similar models have been developed and analyzed by others (Aksimentiev *et al.*, 2004; Elston *et al.*, 1998; Junge *et al.*, 1997).

Previous work (Long *et al.*, 1998; Wada *et al.*, 1999), summarized in 2000 (Vik *et al.*, 2000), had shown that subunit *a* had five transmembrane spans, with the amino-terminus in the cytoplasm and the carboxy-terminus in the cytoplasm (see also (Valiyaveetil and Fillingame, 1998)). These results placed Arg 210 near the center of the membrane, and other key residues, His 245 and Glu 219

[1] Department of Biological Sciences, Southern Methodist University, Dallas, Texas 75275–0376.

[2] To whom correspondence should be addressed; e-mail: svik@smu.edu.
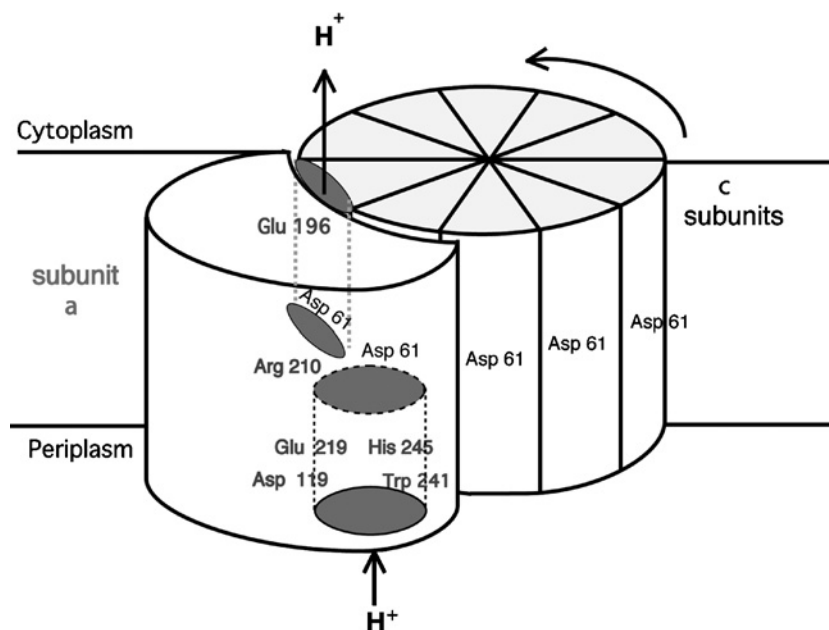
Fig. 1. Model for rotary proton translocation by subunits *a* and *c*. The *c* subunits are modeled as an oligomer of 10 with two subunits at the interface of subunit *a*. The Asp 61 residues of these two subunits are ionized. They interact with the Arg 210 from the *a* subunit. The access channel from the periplasm is formed by the indicated residues within subunit *a*. The access channel to the cytoplasm occurs at the interface of *a* and *c* subunits, near Glu 196.

towards the periplasmic side. Another conserved residue, Glu 196, was shown to be exposed in the cytoplasm. It was also shown that about 35 residues of the amino-terminus are in the periplasm. In general, the ends of the transmembrane spans were not determined, and so further work was done to better define them. Other new studies employed bi-functional cross-linkers to probe subunit-subunit interactions (DeLeon-Rangel *et al.*, 2003; Long *et al.*, 2002; Zhang and Vik, 2003a), and a chemical protease to probe transmembrane packing in subunit *a* (Zhang and Vik, 2003b). These studies used monocysteine substitutions at various positions in subunit *a*, followed by reaction with a sulfhydryl reagent, typically MPB. Subunit *a* has no naturally occurring cysteines (Walker *et al.*, 1984). The results described below are summarized in Fig. 2.

CYTOPLASMIC LOOPS OF SUBUNIT *a*

Subunit *a* contains two loops, in addition to its carboxy-terminus, in the cytoplasm of *E. coli*. The last 10 residues were shown to be dispensable for activity (Vik *et al.*, 1991), and several of these residues could be labeled from the cytoplasmic surface with MPB (Long *et al.*, 1998; Wada *et al.*, 1999). The first cytoplasmic loop, Loop 1-2, contains about 40 amino acids (Long *et al.*, 2002). Thirty-seven residues between 60 and 103

were changed to cysteine individually, and reacted with MPB in preparations of inverted membrane vesicles. All residues tested between 64 and 74 and between 90 and 103 could be labeled, while in a region between residues 75 and 89, about half of the residues were resistant to labeling. These residues are presumably shielded from labeling by protein or lipid. Since this is a rather polar region, including Gln 76, Thr 77, Glu 80, and Ser 89, shielding by lipids seemed unlikely. Cross-linking studies using the photoactivatable TFPAM-3, which reacts with sulfhydryl groups, showed that when residues 74 and 91 were changed to cysteine, subunit *a–b* covalent-heterodimers were formed. Cross-linking through residue 74 was shown not to affect ATP-driven proton translocation, consistent with the existence of an *a–b*$_2$ stator. The role of Glu 80 was examined by construction of several different substitutions. This residue is highly conserved among bacteria and chloroplasts. The most deleterious substitution was lysine, but these effects were likely related to assembly problems. In conclusion, Loop 1–2 is likely to make contacts with one or both *b* subunits, and is not involved in proton translocation.

Loop 3–4 is also a cytoplasmic loop, and it contains about 35 amino acids (Zhang and Vik, 2003a). Forty-one residues between 160 and 206 were change individually to cysteine, and reacted with MPB in preparations of inverted
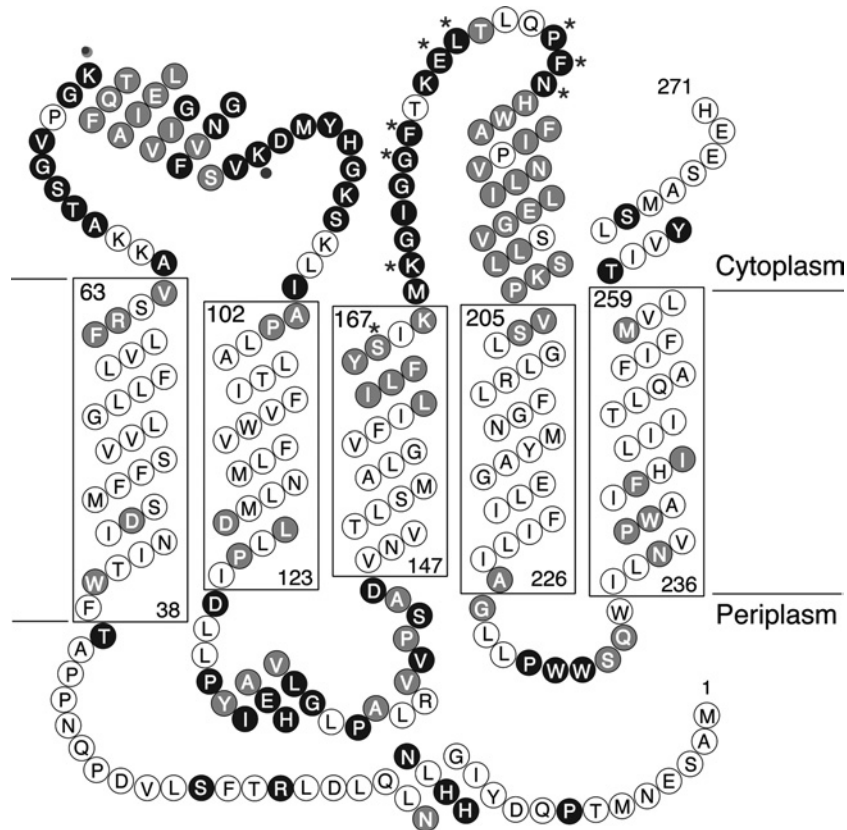
Fig. 2. Transmembrane model of subunit *a*. Residues in black circles are considered surface accessible, since when substituted with cysteine they exhibit significant levels of labeling with MPB. Residues in gray showed little or no labeling with MPB. Residues marked with a dot, K74 and K91, can be cross-linked to the *b* subunit using TFPAM-3, after substitution with cysteine. Residues marked with an asterisk can be cross-linked to *c* subunits with TFPAM-3, after substitution with cysteine.

membrane vesicles. All residues that were tested between 168 and 184, with the exception of Thr 179, were labeled to a significant extent. All residues tested between 185 and 206 labeled very weakly or not at all. Similar to the segment in Loop 1–2, the shielded segment contained several highly conserved polar residues, including Asn 192, Glu 196 and Ser 206. Earlier studies had shown that substitutions of Glu 196 (Vik *et al.*, 1988) and of Asn 192 (Vik *et al.*, 1990) can diminish the rates of ATP-driven proton translocation. Again, the results of cross-linking studies indicated a likely source of the shielding of this segment. TFPAM-3 cross-linking from residues 165, 169, 173, 174, 177, 178, 182, 183, and 184 to *c* subunits demonstrated an extensive region of contact between the two subunits. A likely possibility is that transmembrane span 4 is part of an extended α-helix that makes contact with a *c* subunit, or perhaps fits between two *c* subunits. This is reasonable since *c* subunits are known to have

α-helices of more than 30 amino acids (Girvin *et al.*, 1998; Meier *et al.*, 2005; Stock *et al.*, 1999).

PERIPLASMIC LOOPS OF SUBUNIT *a*

Subunit *a* contains two loops, in addition to its amino-terminus, in the periplasm of *E. coli*. Seven out of eight residues tested in the first 37, were shown to be labeled in the periplasm (Long *et al.*, 1998; Patterson *et al.*, 1999; Wada *et al.*, 1999). Such studies can be carried out in whole cells, with (Wada *et al.*, 1999) or without (Zhang *et al.*, 2003) a permeabilizing agent, polymyxin B non-apeptide, using MPB as the labeling reagent. Two conserved histidine residues are present in this segment, and mutagenesis seems to impact assembly of the *a* subunit (Patterson *et al.*, 1999). This work is currently being followed up in our laboratory (R. R. Ishmukhametov and S. B. Vik). In contrast, Loop 4–5 appears to be highly

shielded. Only three residues, 230–232, showed modest levels of labeling, while nine other residues in this region were not labeled (Wada *et al.*, 1999).

Periplasmic Loop 2–3 consists of about twenty amino acids (Zhang and Vik, 2003b). Twenty-one residues between 119 and 146 were changed to cysteine and reacted with MPB in whole cells. About half of the tested residues between 124 and 146 were shown to be labeled, with no significant segment shielded. Functional analysis of three Cys mutants, Asp 119, Asp 124, and Asp 146, revealed that only Asp 119 of transmembrane span 2 appears to be an important residue (DeLeon-Rangel *et al.*, 2003). Cross-linking studies using monocysteine mutants in this region has shown that several residues provide sites that can be cross-linked to *b* subunits, using benzophenone-maleimide (R. R. Ishmukhametov and S. B. Vik, unpublished results). In conclusion, it appears that periplasmic Loop 2–3 contains no functionally important residues, but that it is in close proximity to the amino-terminal regions of the *b* subunits. Since both cytoplasmic Loop 1–2 and periplasmic Loop 2–3 can be cross-linked to *b* subunits, it is likely that transmembrane span 2 of subunit *a* makes contact with the transmembrane spans of subunit *b*.

HELIX PACKING OF SUBUNIT *a*

The arrangement of transmembrane helices of subunit *a* was investigated by use of a chemical protease (Zhang and Vik, 2003b). Monocysteine mutants were used as sites for attachment of a bi-functional reagent, 5-(-bromoacetamido)-1,10-phenanthroline-copper. After oxidation, the reagent can catalyze the fragmentation of a nearby polypeptide chain (Wu *et al.*, 1995). The site of fragmentation is identified by comparing the size of the fragment with truncated subunit *a* fragments of known size. In this way the proximity of a given residue with a different transmembrane helix can be discovered. Our study revealed only two positions that led to fragmentation of subunit *a*, Trp 241 and Asp 44 or Ile 43. In all three cases, the products were about the same size, and corresponded to fragmentation at the periplasmic end of transmembrane helix 2. The results with Trp 241 are consistent with the finding that D119H is an effective suppressor of H245C (Valiyaveetil and Fillingame, 1998). The limited number of monocysteine substitutions that led to fragmentation is probably a consequence of the function of subunit *a*. Since it is involved in the transport of protons, the helices are likely to be packed closely, and might therefore have little room to accommodate the proteolysis reagent. It is notable that the greatest cleavage was obtained by a cysteine substitution for tryptophan, the largest amino acid.

CURRENT STUDIES

Current studies are using the rapid purification procedure recently developed in our laboratory (Ishmukhametov *et al.*, 2005). In the case of the amino-terminus, it will be possible to distinguish between its importance in assembly, and its possible role in function. Other studies will use reconstitution methods to examine the role of particular roles of important amino acid residues of the *a* subunit.

ACKNOWLEDGMENT

REFERENCES

Aksimentiev, A., Balabin, I. A., Fillingame, R. H., and Schulten, K. (2004). *Biophys. J.* **86**, 1332–1344.

Angevine, C. M., and Fillingame, R. H. (2003). *J. Biol. Chem.* **278**, 6066–6074.

Angevine, C. M., Herold, K. A., and Fillingame, R. H. (2003). *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13179–13183.

Bragg, P. D., and Hou, C. (1975). *Arch. Biochem. Biophys.* **167**, 311–321.

Cain, B. D., and Simoni, R. D. (1989). *J. Biol. Chem.* **264**, 3292–3300.

Capaldi, R. A., and Aggeler, R. (2002). *Trends Biochem. Sci.* **27**, 154–160.

DeLeon-Rangel, J., Zhang, D., and Vik, S. B. (2003). *Arch. Biochem. Biophys.* **418**, 55–62.

Elston, T., Wang, H., and Oster, G. (1998). *Nature* **391**, 510–513.

Foster, D. L., and Fillingame, R. H. (1982). *J. Biol. Chem.* **257**, 2009–2015.

Girvin, M. E., Rastogi, V. K., Abildgaard, F., Markley, J. L., and Fillingame, R. H. (1998). *Biochemistry* **37**, 8817–8824.

Ishmukhametov, R. R., Galkin, M. A., and Vik, S. B. (2005). *Biochim. Biophys. Acta* **1706**, 110–116.

Junge, W., Lill, H., and Engelbrecht, S. (1997). *Trends Biochem. Sci.* **22**, 420–423.

Lightowlers, R. N., Howitt, S. M., Hatch, L., Gibson, F., and Cox, G. B. (1987). *Biochim. Biophys. Acta* **894**, 399–406.

Long, J. C., DeLeon-Rangel, J., and Vik, S. B. (2002). *J. Biol. Chem.* **277**, 27288–27293.

Long, J. C., Wang, S., and Vik, S. B. (1998). *J. Biol. Chem.* **273**, 16235–16240.

Meier, T., Polzer, P., Diederichs, K., Welte, W., and Dimroth, P. (2005). *Science* **308**, 659–662.

Patterson, A. R., Wada, T., and Vik, S. B. (1999). *Arch. Biochem. Biophys.* **368**, 193–197.

Stock, D., Leslie, A. G., and Walker, J. E. (1999). *Science* **286**, 1700–1705.

Valiyaveetil, F. I., and Fillingame, R. H. (1998). *J. Biol. Chem.* **273**, 16241–16247.

Vik, S. B., and Antonio, B. J. (1994). *J. Biol. Chem.* **269**, 30364–30369.

Vik, S. B., Cain, B. D., Chun, K. T., and Simoni, R. D. (1988). *J. Biol. Chem.* **263**, 6599–6605.

Vik, S. B., Lee, D., Curtis, C. E., and Nguyen, L. T. (1990). *Arch. Biochem. Biophys.* **282**, 125–131.

Vik, S. B., Lee, D., and Marshall, P. A. (1991). *J. Bacteriol.* **173**, 4544–4548.

Vik, S. B., Long, J. C., Wada, T., and Zhang, D. (2000). *Biochim. Biophys. Acta* **1458**, 457–466.

Vik, S. B., Patterson, A. R., and Antonio, B. J. (1998). *J. Biol. Chem.* **273**, 16229–16234.

Wada, T., Long, J. C., Zhang, D., and Vik, S. B. (1999). *J. Biol. Chem.* **274**, 17353–17357.

Walker, J. E., Saraste, M., and Gay, N. J. (1984). *Biochim. Biophys. Acta* **768**, 164–200.

Wu, J., Perrin, D. M., Sigman, D. S., and Kaback, H. R. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9186–9190.

Zhang, D., and Vik, S. B. (2003a). *J. Biol. Chem.* **278**, 12319–12324.

Zhang, D., and Vik, S. B. (2003b). *Biochemistry* **42**, 331–337.

Zhang, W., Bogdanov, M., Pi, J., Pittard, A. J., and Dowhan, W. (2003). *J. Biol. Chem.* **278**, 50128–50135.