

## **ESCHERICHIA COLI ATP SYNTHASE (F-ATPase): CATALYTIC SITE AND REGULATION OF H<sup>+</sup> TRANSLOCATION**

BY MASAMITSU FUTAI\*, ATSUKO IWAMOTO, HIROSHI OMOTE, YUJI ORITA, KOUICHIROU SHIN, ROBERT K. NAKAMOTO AND MASATOMO MAEDA

*Department of Organic Chemistry and Biochemistry, Institute of Scientific and Industrial Research, Osaka University, Osaka 567, Japan*

### **Summary**

We discuss our recent results on the *Escherichia coli* F-ATPase, in particular its catalytic site in the  $\beta$  subunit and regulation of H<sup>+</sup> transport by the  $\gamma$  subunit. Affinity labelling experiments suggest that  $\beta$ Lys-155 in the glycine-rich sequence is near the  $\gamma$ -phosphate moiety of ATP bound at the catalytic site. The enzyme loses activity upon introduction of missense mutations in  $\beta$ Lys-155 or  $\beta$ Thr-156 and changes catalytic properties upon introduction of other mutations. By analysis of mutations and their pseudo revertants, residues  $\beta$ Ser-174,  $\beta$ Glu-192 and  $\beta$ Val-198 were found to be located near the glycine-rich sequence. The combined approaches of chemical labelling and genetics have been fruitful in visualizing the structure of the catalytic site. Analysis of mutations in the  $\gamma$  subunit suggests that this subunit has an essential role in coupling catalysis with proton translocation.

### **Introduction**

The ATP synthase (or F-ATPase) catalyzes ATP synthesis or hydrolysis coupled with proton translocation (for reviews, see Fillingame, 1990; Futai *et al.* 1989, 1991; Senior, 1990). The catalytic sector F<sub>1</sub> (F<sub>1</sub>-ATPase) is composed of five different subunits:  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ . The  $\beta$  and  $\alpha$  subunits have homologies with the A ( $72 \times 10^3 M_r$ ) and B ( $54 \times 10^3 M_r$ ) subunits, respectively, of the V-ATPase (for a review, see Forgac, 1989). Furthermore the F<sub>0</sub> sector *c* subunit, which forms the proton pathway in the F-ATPase, is similar to the proteolipid of the V-ATPase (Mandel *et al.* 1988; Hanada *et al.* 1991). Thus, the two types of ATPase are likely to have many common structural and mechanistic features (Hanada *et al.* 1990).

The F-ATPase of *Escherichia coli* is similar to those found in mitochondria and chloroplasts. Over the past 10 years, structure–function relationships of the bacterial enzyme have been extensively studied by a combination of affinity labelling (chemical modification) and mutational analysis. These approaches identified many of the residues making up the catalytic site in the  $\beta$  subunit and the proton pathway. More recently, the role of the  $\gamma$  subunit in coupling ATP hydrolysis/synthesis to proton translocation has been recognized. In this article we discuss our studies in defining the catalytic site near the ATP  $\gamma$ -phosphate moiety and the role(s) of the  $\gamma$  subunit.

\*To whom reprint requests should be addressed.

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### The glycine-rich sequence

The glycine-rich sequence, Gly-X-X-X-X-Gly-Lys-Thr (Fig. 1A), is found in many nucleotide binding proteins, including the  $\beta$  subunit of F-ATPase, the A subunit of V-ATPase, adenylate kinase and p21 *ras* protein (Walker *et al.* 1984). Crystallographic studies of adenylate kinase and *ras* proteins have shown that this sequence forms a loop structure between an  $\alpha$  helix and a  $\beta$  sheet (Dreusicke *et al.* 1988; Milburn *et al.* 1990). In the *E. coli*  $\beta$  subunit, the glycine-rich sequence corresponds to positions 149–156 (Gly-Gly-Ala-Gly-Val-Gly-Lys-Thr). We have been interested in the roles of the sequence in the catalytic mechanism of F<sub>1</sub> ever since we obtained the primary structure of the  $\beta$  subunit (Kanazawa *et al.* 1982).

#### *Binding of adenosine triphosphopyridoxal to $\beta$ Lys-155*

The F<sub>1</sub>-ATPase was shown to lose its activity after binding 1 mole of adenosine triphosphopyridoxal (AP<sub>3</sub>-PL), and this inhibition was prevented by adding ATP (Noumi *et al.* 1987). Approximately 60% of AP<sub>3</sub>-PL was in the  $\alpha$  subunit and 40% in the  $\beta$  subunit. Lys-201 of the  $\alpha$  subunit and Lys-155 of the  $\beta$  subunit were target residues, indicating that binding of AP<sub>3</sub>-PL to either residue inhibited activity (Tagaya *et al.* 1988). Addition of Mg<sup>2+</sup> decreased the AP<sub>3</sub>-PL concentration required for inhibition and shifted the labelling predominantly to the  $\beta$  subunit ( $\beta$ Lys-155 and  $\beta$ Lys-201) (Ida *et al.* 1991). These results suggest that, like the  $\beta$ Lys-201 and  $\alpha$ Lys-201 residues, the  $\beta$ Lys-155 residue is near the  $\gamma$ -phosphate moiety of ATP. It should be noted that the corresponding lysine residues in the glycine-rich sequences of adenylate kinase (Tagaya *et al.* 1987) and p21 *ras* protein (Ohmi *et al.* 1988) were labelled with adenosine diphosphopyridoxal and guanosine triphosphopyridoxal, respectively. The pyridoxal nucleotide derivatives have proved useful as affinity analogues for labelling the lysine residue in the glycine-rich sequence of nucleotide binding proteins.

The results of our labelling experiments stimulated us to introduce mutations into  $\alpha$ Lys-201 (Ida *et al.* 1991). The  $\alpha$  subunit mutants ( $\alpha$ Lys-201→Glu or  $\alpha$ Lys-201 deletion) were active in oxidative phosphorylation. The purified mutant enzymes had lower multisite catalysis than the wild-type enzyme but were similar to it in single-site (uni-site) catalysis. These results suggest that  $\alpha$ Lys-201, or residues in its vicinity, is important for catalytic cooperativity but not for catalysis itself.

#### *Mutations in the glycine-rich sequence*

The glycine-rich sequence of the  $\beta$  subunit could be replaced by that of the *ras* protein, even though three residues in the sequences are different (Takeyama *et al.* 1990); the mutant enzyme with the *ras* protein sequence (Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser, residues 10–17) had approximately 40% of the wild-type ATPase activity and was active in ATP synthesis. These results suggested that the glycine-rich sequences of the two proteins are similar in structure and function. In contrast, the sequence of adenylate kinase (Gly-Gly-Pro-Gly-Ser-Gly-Lys-Gly-Thr, residues 15–23) could not function in the  $\beta$  subunit. Significant differences between the sequences of the two proteins are apparent; in contrast to the  $\beta$  subunit, adenylate kinase has a glycine insertion between the

lysine and threonine residues, which probably changes the position and projection of the side chain of the  $\beta$ Thr-156 residue.

The above results suggest that  $\beta$ Thr-156 itself is essential for catalysis. As expected,  $\beta$ Thr-156 $\rightarrow$ Cys and  $\beta$ Thr-156 $\rightarrow$ Ala mutant enzymes had neither steady-state (multi-site catalysis) nor single-site (uni-site catalysis) ATPase activities (Iwamoto *et al.* 1991; Omote *et al.* 1992). Conversely,  $\beta$ Thr-156 could be replaced by Ser without loss of activity, consistent with the activity of the *ras*-like  $\beta$  mutant ( $\beta$ Thr-156 corresponds to *ras* Ser residue). Furthermore, the  $\beta$ Lys-155 $\rightarrow$ Ala or Ser mutant had no uni- and multi-site activity, indicating that this residue is also essential for catalysis (H. Omote, M. Maeda and M. Futai, in preparation).

The  $\beta$ Gly-149 $\rightarrow$ Ser and  $\beta$ Gly-150 $\rightarrow$ Ser mutant enzymes had essentially normal activities (Iwamoto *et al.* 1991), whereas  $\beta$ Ala-151 $\rightarrow$ Val (Hsu *et al.* 1987) and  $\beta$ Ala-151 $\rightarrow$ Pro (Takeyama *et al.* 1990) mutants had approximately 6 and 200%, respectively, of the wild-type membrane ATPase activity. Replacement of the  $\beta$ Ala-151 residue may affect the orientation of catalytically essential residues such as  $\beta$ Lys-155 and  $\beta$ Thr-156 and change the kinetic properties of the enzyme.

The V-ATPases and F-ATPases so far studied can be differentiated simply by their inhibitor sensitivities (Forgac, 1989): azide specifically inhibits F-ATPase, whereas *N*-ethyl maleimide specifically inhibits V-ATPases. However, after a single amino acid substitution in the glycine-rich sequence, the inhibitor sensitivity of the F-ATPase became similar to that of the V-ATPase. Strikingly, a  $\beta$ Gly-149 $\rightarrow$ Ser mutation increased the apparent  $K_i$  for azide more than 100-fold; the concentrations of azide required for 50% inhibition of the ATPase activity of the wild type and  $\beta$ Ser-149 mutant were 0.034 and 4.3 mmol l<sup>-1</sup>, respectively (Iwamoto *et al.* 1991). The  $\beta$ Ser-150 mutant enzyme was not as azide-resistant, being about fourfold less sensitive than the wild type. The A subunit of V-ATPase has a cysteine residue corresponding to position 153 of the  $\beta$  subunit of F-ATPase. The glycine-rich sequences of the A subunits of *Neurospora crassa* and carrot are Gly-Ala-Phe-Gly-Cys-Gly-Lys-Thr (Bowman *et al.* 1988; Zimniak *et al.* 1988). The  $\beta$ Val-153 $\rightarrow$ Cys mutant of F-ATPase is as sensitive to *N*-ethyl maleimide as is the wild type of V-ATPase. A simple but important lesson from these results is that unknown enzymes cannot be classified simply by their sensitivity to inhibitors, since inhibitor sensitivities can be altered by single amino acid substitutions.

### Amino acid residues located near the glycine-rich sequence

Residues near the binding site for the  $\gamma$ -phosphate moiety of ATP can be mapped by genetic procedures: pseudo revertants of the mutants in the glycine-rich sequence may yield two closely related amino acid residues (the first from the original mutation and the second from a pseudo reversion). In contrast, two residues can also be functionally or structurally related if one mutation is suppressed by the second mutation in the glycine-rich sequence.

The  $\beta$ Ser-174 $\rightarrow$ Phe mutant is one of the early mutants isolated in our laboratory, and has about 6% of the wild-type membrane ATPase activity (Kanazawa *et al.* 1980). We found that the effect of this mutation was suppressed by a second mutation in the  $\beta$  gene,

Gly149→Ser (Iwamoto *et al.* 1991). To confirm the suppression, the two mutations were introduced separately or together into pBWU13, which carries the entire *unc (atp)* operon. Other replacements at residue 149 could suppress the  $\beta$ Phe-174 mutation; the  $\beta$ Phe-174/ $\beta$ Ala-149 mutant was similar in activity to the wild type and the  $\beta$ Phe-174/ $\beta$ Cys-149 mutant had a low but significantly higher membrane ATPase activity than the  $\beta$ Phe-174 mutant alone (A. Iwamoto, M.-Y. Park, M. Maeda and M. Futai, in preparation). It is also noteworthy that the single  $\beta$ Gly-149→Cys mutation resulted in a defective enzyme. In contrast, the  $\beta$ Gly-149→Thr or  $\beta$ Gly-150→Ser mutations could not suppress the effect of the  $\beta$ Phe-174 mutation. It would appear that suppression of the effect of the  $\beta$ Ser-174→Phe mutation requires residues smaller than Thr. These results strongly suggest that the  $\beta$ Gly-149 and  $\beta$ Ser-174 residues interact functionally and that both are located near the  $\gamma$ -phosphate moiety of ATP.

We have also isolated pseudo revertants of the  $\beta$ Cys-149 mutant, and found that either the  $\beta$ Val-198→Ala or the  $\beta$ Glu-192→Val mutation could suppress the effect of the  $\beta$ Cys-149 mutation. Thus, the  $\beta$ Glu-192 and  $\beta$ Val-198 residues also interact functionally with the  $\beta$ Gly-149 residue. It is reasonable to conclude that  $\beta$ Gly-149 and  $\beta$ Val-198 are located close together, because  $\beta$ Lys-201 (three residues downstream of  $\beta$ Val-198) was found to be near the binding site of the  $\gamma$ -phosphate moiety of ATP together with  $\beta$ Lys-155, as shown by labelling with AP<sub>3</sub>-PL (Ida *et al.* 1991). Consistent with the location of the  $\beta$ Glu-192 residue being near the catalytic site, binding of dicyclohexylcarbodiimide (DCCD) to this residue completely inhibited enzyme activity (Yoshida *et al.* 1982). These results are summarized in a model of the catalytic site near the  $\gamma$ -phosphate moiety of ATP (Fig. 1B).

### Role of the $\gamma$ subunit in the regulation of H<sup>+</sup> transport

Like other ion-motive ATPases, ATP hydrolysis/synthesis at the catalytic site of F-ATPase is tightly coupled with proton transport through the F<sub>o</sub> membrane sector. The mechanism of this coupling is not fully understood, but we think that the  $\gamma$  subunit may have regulatory functions in coupling. The roles of the  $\gamma$  subunit and its amino acid residues are less well understood than those of the  $\beta$  subunit. The chloroplast  $\gamma$  subunit has a unique domain containing two cysteine residues in which a disulfide to sulfhydryl transition activates the enzyme (Miki *et al.* 1988; Inohara *et al.* 1991), whereas such domains are not found in the subunits from mitochondria or bacteria. However, the *E. coli*  $\gamma$  subunit may have regulatory role(s) because the subunit is required, along with the  $\alpha$  and  $\beta$  subunits, to reconstitute the minimal ATPase complex. We introduced mutations in the carboxyl terminal region between  $\gamma$ Gln-269 and  $\gamma$ Thr-277 because the termination mutant ( $\gamma$ Thr-277→end) was similar in activity to the wild type, whereas the  $\gamma$ Gln-269→end mutant had no ATPase activity (Miki *et al.* 1986; Iwamoto *et al.* 1990). The results of replacing the  $\gamma$ Gln-269,  $\gamma$ Thr-273 and  $\gamma$ Glu-275 residues suggested that they are required for normal catalytic activity.

Interestingly, the ATPase activities of mutant membranes did not correlate with their abilities for ATP-driven H<sup>+</sup> translocation: membranes of the  $\gamma$ Gln-269→Leu,  $\gamma$ Glu-275→Lys,  $\gamma$ Thr-277→end mutants and a frameshift (downstream of  $\gamma$ Thr-277) mutant



43 residues of the carboxyl terminus. These results clearly suggest that the two ends of the  $\gamma$  subunit participate in coupling between ATP synthesis/hydrolysis and proton translocation.

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### References

- BOWMAN, E. J., TENNEY, K. AND BOWMAN, B. J. (1988). Isolation of genes encoding the *Neurospora* vacuolar ATPase: analysis of *vma-1* encoding the 67-kDa subunit reveals homology to other ATPases. *J. biol. Chem.* **263**, 13994–14001.
- DREUSICKE, D., KARPLUS, P. A. AND SCHULZ, G. A. (1988). Refined structure of porcine cytosolic adenylate kinase at 2 Å resolution. *J. molec. Biol.* **199**, 359–371.
- FILLINGAME, R. H. (1990). Molecular mechanics of ATP synthesis by  $F_1F_0$ -type  $H^+$ -transporting ATP synthases. In *The Bacteria*, vol. XII, pp. 345–391. New York, Tokyo: Academic Press.
- FORGAC, M. (1989). Structure and function of vacuolar class of ATP-driven proton pumps. *Physiol. Rev.* **69**, 765–796.
- FUTAI, M., HANADA, H., MORIYAMA, Y. AND MAEDA, M. (1991). Proton translocating ATP synthase ( $F_1F_0$ ): understanding its molecular structure and function. In *New Era of Bioenergetics* (ed. Y. Mukohata), pp. 73–108. New York, Tokyo: Academic Press.
- FUTAI, M., NOUMI, T. AND MAEDA, M. (1989). ATP synthase ( $H^+$ -ATPase); results by combined biochemical and molecular biological approaches. *A. Rev. Biochem.* **58**, 111–136.
- HANADA, H., HASEBE, M., MORIYAMA, Y., MAEDA, M. AND FUTAI, M. (1991). Molecular cloning of cDNA encoding the 16 kDa subunit of vacuolar  $H^+$ -ATPase from mouse cerebellum. *Biochem. biophys. Res. Commun.* **176**, 1062–1067.
- HANADA, H., MORIYAMA, Y., MAEDA, M. AND FUTAI, M. (1990). Kinetic studies of chromaffin granule  $H^+$ -ATPase and effects of bafilomycin A<sub>1</sub>. *Biochem. biophys. Res. Commun.* **170**, 873–878.
- HSU, S.-Y., NOUMI, T., TAKEYAMA, M., MAEDA, M., ISHIBASHI, S. AND FUTAI, M. (1987).  $\beta$ -subunit of *Escherichia coli*  $F_1$ -ATPase. An amino acid replacement within a conserved sequence (G-X-X-X-X-G-K-T/S) of nucleotide-binding proteins. *FEBS Lett.* **218**, 222–226.
- IDA, K., NOUMI, T., MAEDA, M., FUKUI, T. AND FUTAI, M. (1991). Catalytic site of  $F_1$ -ATPase of *Escherichia coli*: Lys-155 and Lys-201 of the  $\beta$  subunit are located near the  $\gamma$ -phosphate group of ATP in the presence of  $Mg^{2+}$ . *J. biol. Chem.* **266**, 5424–5429.
- INOHARA, N., IWAMOTO, A., MORIYAMA, Y., SHIMOMURA, S., MAEDA, M. AND FUTAI, M. (1991). Two genes, *atpC1* and *atpC2*, for the  $\gamma$  subunit of *Arabidopsis thaliana* chloroplast ATP synthase. *J. biol. Chem.* **266**, 7333–7338.
- IWAMOTO, A., MIKI, J., MAEDA, M. AND FUTAI, M. (1990).  $H^+$ -ATPase  $\gamma$  subunit from *Escherichia coli*: role of the conserved carboxyl terminal region. *J. biol. Chem.* **265**, 5043–5048.
- IWAMOTO, A., OMOTE, H., HANADA, H., TOMIOKA, N., ITAI, A., MAEDA, M. AND FUTAI, M. (1991). Mutations in Ser-174 and the glycine-rich sequence (Gly-129, Gly-150, and Thr-156) in the  $\beta$  subunit of *Escherichia coli*  $H^+$ -ATPase. *J. biol. Chem.* **266**, 16350–16355.
- KANAZAWA, H., HORIUCHI, Y., TAKAGI, M., ISHINO, Y. AND FUTAI, M. (1980). Coupling factor  $F_1$  ATPase with defective  $\beta$  subunit from a mutant of *Escherichia coli*. *J. Biochem., Tokyo* **88**, 695–703.
- KANAZAWA, H., KAYANO, T., KIYASU, T. AND FUTAI, M. (1982). Nucleotide sequence of the genes for  $\beta$  and  $\epsilon$  subunits of proton-translocating ATPase from *Escherichia coli*. *Biochem. biophys. Res. Commun.* **105**, 1257–1264.
- MANDEL, M., MORIYAMA, Y., HULMES, J. D., PAN, Y.-C. E., NELSON, H. AND NELSON, N. (1988). cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of  $H^+$ -ATPases. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5521–5524.
- MIKI, J., MAEDA, M., MUKOHATA, Y. AND FUTAI, M. (1988). The  $\gamma$ -subunit of ATP synthase from spinach chloroplasts. Primary structure deduced from the cloned cDNA sequence. *FEBS Lett.* **232**, 221–226.
- MIKI, J., TAKEYAMA, M., NOUMI, T., KANAZAWA, H., MAEDA, M. AND FUTAI, M. (1986). *Escherichia coli*

- H<sup>+</sup>-ATPase: loss of the carboxyl terminal region of the  $\gamma$ -subunit causes defective assembly of the F<sub>1</sub> portion. *Archs Biochem. Biophys.* **241**, 458–464.
- MILBURN, M. V., TONG, L., DE VOS, A. M., BRUNGER, A., YAMAIZUMI, Z., NISHIMURA, S. AND KIM, S.-H. (1990). Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic *ras* proteins. *Science* **247**, 939–945.
- NOUMI, T., TAGAYA, M., MIKI-TAKEDA, K., MAEDA, M., FUKUI, T. AND FUTAI, M. (1987). Loss of unisite and multisite catalysis by *Escherichia coli* F<sub>1</sub> through modification with adenosine tri- or tetraphosphopyridoxal. *J. biol. Chem.* **262**, 7686–7692.
- OHMI, N., HOSHINO, M., TAGAYA, M., FUKUI, T., KAWAKITA, M. AND HATTORI, S. (1988). Affinity labelling of *ras* oncogene product p21 with guanosine diphospho- and triphosphopyridoxals. *J. biol. Chem.* **263**, 14261–14266.
- OMOTE, H., MAEDA, M. AND FUTAI, M. (1992). Effects mutations of conserved lys-155 and thr-156 residues in the phosphate binding glycine-rich sequence of the F<sub>1</sub>-ATPase  $\beta$  subunit of *Escherichia coli*. *J. biol. Chem.* **267** (in press).
- SENIOR, A. E. (1990). The proton-translocating ATPase of *Escherichia coli*. *A. Rev. biophys. Chem.* **19**, 7–41.
- SHIN, K., NAKAMOTO, R. K., MAEDA, M. AND FUTAI, M. (1992). F<sub>0</sub>F<sub>1</sub>-ATPase  $\gamma$  subunit mutations perturb the coupling between catalysis and transport. *J. biol. Chem.* **267** (in press).
- TAGAYA, M., NOUMI, T., NAKANO, K., FUTAI, M. AND FUKUI, T. (1988). Identification of  $\alpha$ -subunit Lys-210 and  $\beta$ -subunit Lys-155 at the ATP-binding sites in *Escherichia coli* F<sub>1</sub>-ATPase. *FEBS Lett.* **233**, 347–351.
- TAGAYA, M., YAGAMI, T. AND FUKUI, T. (1987). Affinity labelling of adenylate kinase with adenosine diphosphopyridoxal: presence of Lys-21 in the ATP-binding site. *J. biol. Chem.* **262**, 8257–8261.
- TAKEYAMA, M., IHARA, K., MORIYAMA, Y., NOUMI, T., IDA, K., TOMIOKA, N., ITAI, A., MAEDA, M. AND FUTAI, M. (1990). The glycine-rich sequence of the  $\beta$  subunit of *Escherichia coli* H<sup>+</sup>-ATPase is important for activity. *J. biol. Chem.* **265**, 21279–21284.
- WALKER, J. E., SARASTE, M. AND GAY, N. J. (1984). The *unc* operon. Nucleotide sequence, regulation and structure of ATP-synthase. *Biochim. biophys. Acta* **768**, 164–200.
- YOSHIDA, M., ALLISON, W. S., ESCH, F. S. AND FUTAI, M. (1982). The specificity of carboxyl group modification during the inactivation of the *Escherichia coli* F<sub>1</sub>-ATPase with dicyclohexyl [C<sup>14</sup>]carbodiimide. *J. biol. Chem.* **257**, 10033–10037.
- ZIMNIAK, L., DITTRICH, P., GOGARTEN, J. P., KIBAK, H. AND TAIZ, L. (1988). The cDNA sequence of the 69-kDa subunit of the carrot vacuolar H<sup>+</sup>-ATPase. *J. biol. Chem.* **263**, 9102–9112.

