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ESCHERICHIA COLI ATP SYNTHASE (F-ATPase): CATALYTIC SITE AND REGULATION OF H^+ 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 β subunit and regulation of H^+ transport by the γ subunit. Affinity labelling experiments suggest that βLys-155 in the glycine-rich sequence is near the γ-phosphate moiety of ATP bound at the catalytic site. The enzyme loses activity upon introduction of missense mutations in βLys-155 or βThr-156 and changes catalytic properties upon introduction of other mutations. By analysis of mutations and their pseudo revertants, residues βSer-174, βGlu-192 and βVal-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 γ 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_1 (F_1-ATPase) is composed of five different subunits: α, β, γ, δ and ϵ. The β and α subunits have homologies with the *A* (72×10^3 M_r) and *B* (54×10^3 M_r) subunits, respectively, of the V-ATPase (for a review, see Forgac, 1989). Furthermore the F_o 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 β subunit and the proton pathway. More recently, the role of the γ 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 γ-phosphate moiety and the role(s) of the γ 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 β 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 α helix and a β sheet (Dreusicke *et al.* 1988; Milburn *et al.* 1990). In the *E. coli* β 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_1 ever since we obtained the primary structure of the β subunit (Kanazawa *et al.* 1982).

Binding of adenosine triphosphopyridoxal to βLys-155

The F_1-ATPase was shown to lose its activity after binding 1 mole of adenosine triphosphopyridoxal (AP_3-PL), and this inhibition was prevented by adding ATP (Noumi *et al.* 1987). Approximately 60% of AP_3-PL was in the α subunit and 40% in the β subunit. Lys-201 of the α subunit and Lys-155 of the β subunit were target residues, indicating that binding of AP_3-PL to either residue inhibited activity (Tagaya *et al.* 1988). Addition of Mg^{2+} decreased the AP_3-PL concentration required for inhibition and shifted the labelling predominantly to the β subunit (βLys-155 and βLys-201) (Ida *et al.* 1991). These results suggest that, like the βLys-201 and αLys-201 residues, the βLys-155 residue is near the γ-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 αLys-201 (Ida *et al.* 1991). The α subunit mutants (αLys-201$\rightarrow$Glu or αLys-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 αLys-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 β 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 β subunit. Significant differences between the sequences of the two proteins are apparent; in contrast to the β 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 βThr-156 residue.

The above results suggest that βThr-156 itself is essential for catalysis. As expected, βThr-156→Cys and βThr-156→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, βThr-156 could be replaced by Ser without loss of activity, consistent with the activity of the *ras*-like β mutant (βThr-156 corresponds to *ras* Ser residue). Furthermore, the βLys-155→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 βGly-149→Ser and βGly-150→Ser mutant enzymes had essentially normal activities (Iwamoto *et al.* 1991), whereas βAla-151→Val (Hsu *et al.* 1987) and βAla-151→Pro (Takeyama *et al.* 1990) mutants had approximately 6 and 200%, respectively, of the wild-type membrane ATPase activity. Replacement of the βAla-151 residue may affect the orientation of catalytically essential residues such as βLys-155 and βThr-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 βGly-149→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 βSer-149 mutant were 0.034 and 4.3 mmol l^{-1}, respectively (Iwamoto *et al.* 1991). The βSer-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 β 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 βVal-153→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 γ-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 βSer-174→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 β 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 βPhe-174 mutation; the βPhe-174/βAla-149 mutant was similar in activity to the wild type and the βPhe-174/βCys-149 mutant had a low but significantly higher membrane ATPase activity than the βPhe-174 mutant alone (A. Iwamoto, M.-Y. Park, M. Maeda and M. Futai, in preparation). It is also noteworthy that the single βGly-149→Cys mutation resulted in a defective enzyme. In contrast, the βGly-149→Thr or βGly-150→Ser mutations could not suppress the effect of the βPhe-174 mutation. It would appear that suppression of the effect of the βSer-174→Phe mutation requires residues smaller than Thr. These results strongly suggest that the βGly-149 and βSer-174 residues interact functionally and that both are located near the γ-phosphate moiety of ATP.

We have also isolated pseudo revertants of the βCys-149 mutant, and found that either the βVal-198→Ala or the βGlu-192→Val mutation could suppress the effect of the βCys-149 mutation. Thus, the βGlu-192 and βVal-198 residues also interact functionally with the βGly-149 residue. It is reasonable to conclude that βGly-149 and βVal-198 are located close together, because βLys-201 (three residues downstream of βVal-198) was found to be near the binding site of the γ-phosphate moiety of ATP together with βLys-155, as shown by labelling with AP_3-PL (Ida *et al.* 1991). Consistent with the location of the βGlu-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 γ-phosphate moiety of ATP (Fig. 1B).

Role of the γ subunit in the regulation of H^+ 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_0 membrane sector. The mechanism of this coupling is not fully understood, but we think that the γ subunit may have regulatory functions in coupling. The roles of the γ subunit and its amino acid residues are less well understood than those of the β subunit. The chloroplast γ 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* γ subunit may have regulatory role(s) because the subunit is required, along with the α and β subunits, to reconstitute the minimal ATPase complex. We introduced mutations in the carboxyl terminal region between γGln-269 and γThr-277 because the termination mutant (γThr-277→end) was similar in activity to the wild type, whereas the γGln-269→end mutant had no ATPase activity (Miki *et al.* 1986; Iwamoto *et al.* 1990). The results of replacing the γGln-269, γThr-273 and γGlu-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^+ translocation: membranes of the γGln-269→Leu, γGlu-275→Lys, γThr-277→end mutants and a frameshift (downstream of γThr-277) mutant

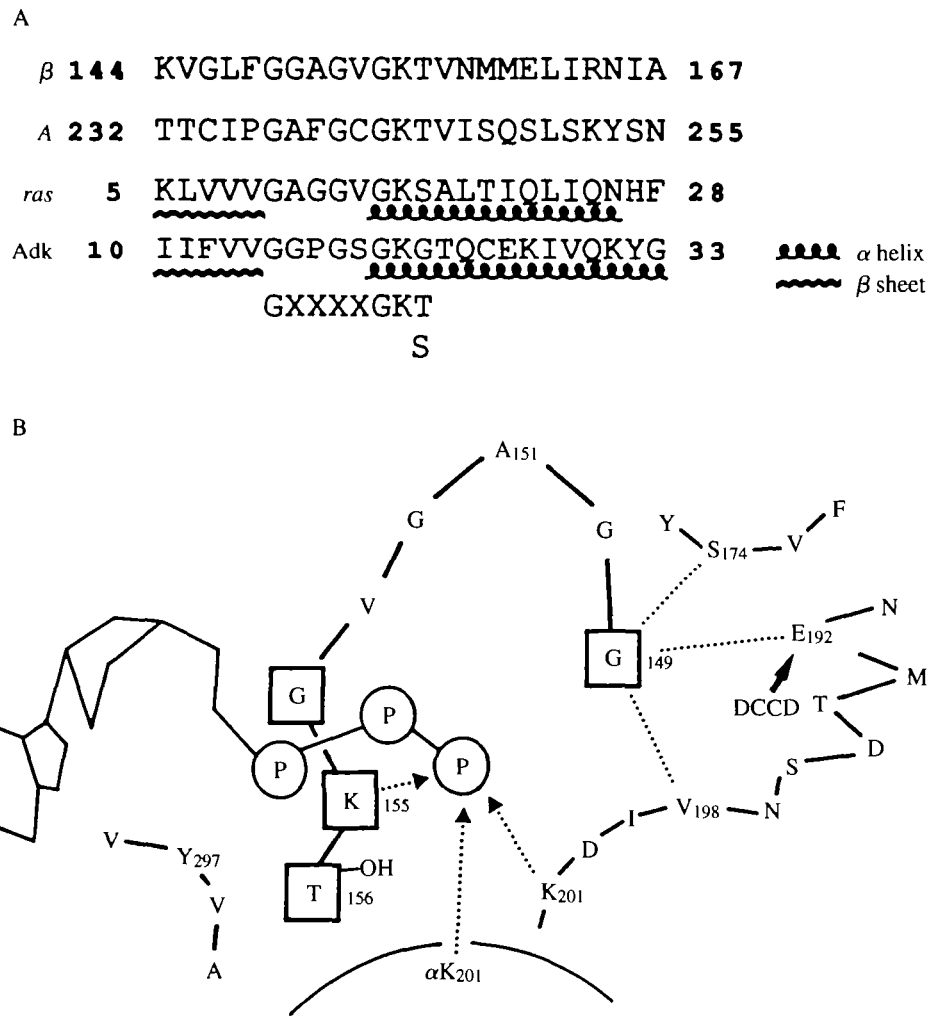

Fig. 1. Glycine-rich sequence and catalytic site of F-ATPase. (A) Alignment of the glycine-rich sequence from the β subunit of *E. coli* F-ATPase (*β*), the *A* subunit of yeast V-ATPase (*A*), the *ras* protein (*ras*) and adenylate kinase (Adk). The secondary structures deduced by crystallography (adenylate kinase, Dreusicke *et al.* 1988; and *ras* protein, Milburn *et al.* 1990) are indicated. (B) A model of the catalytic site near the γ-phosphate moiety of ATP in the β subunit. The combined approaches of affinity labelling and analysis of random and directed mutants and pseudo revertants suggest the amino acid residues shown in or near the catalytic site. See text for details.

had similar low ATPase activities, but formed different degrees of electrochemical gradient of protons. The four mutations had different effects on the coupling between ATP hydrolysis and H^+ translocation. We recently found that the γMet-23→Lys or Arg mutation resulted in an uncoupled enzyme: both enzymes showed membrane ATPase activities similar to that of wild type, but demonstrated substantially lower ATP-dependent H^+ translocation and *in vivo* ATP synthesis (Shin *et al.* 1992). Furthermore, it is of interest that the effect of the γLys-23 mutation was suppressed by mutations within

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

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