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Minireview

A Glycine-Rich Sequence in the Catalytic Site of F-Type ATPase

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Affinity labeling and genetic studies on the glycine-rich sequence of the β subunit of *E. coli* F-type ATPase are discussed. A model of the structure of the enzyme near the γ phosphate moiety is proposed.

KEY WORDS: β subunit of F-type ATPase; glycine-rich sequence.

INTRODUCTION

F-type ATPase (F_0F_1) synthesizes ATP using an electrochemical gradient of protons. The F_1 portion (or F_1-ATPase) is formed from the α, β, γ, δ, and ε subunits, and catalyzes ATP hydrolysis. Two kinetic types (unisite and multisite catalyses) of ATP hydrolysis by F_1 have been reported: the rate of hydrolysis of ATP by unisite (or single-site) catalysis (ATP/F_1 ratio less than 1) is at least 10^5-fold less than that of multisite (or steady-state) catalysis in the presence of excess ATP (Cross *et al.*, 1982: Grubmeyer *et al.*, 1982). The catalytic cooperativity in multisite catalysis is due to the presence of three catalytic sites in F_1. The F-type ATPase of *Escherichia coli* can be studied by the combined approaches of introducting mutations and chemical modifications (or affinity labeling) (for review see: Fillingame, 1990; Futai *et al.*, 1989, 1991; Senior, 1990).These approaches have yielded much information on the residues in (or near) the catalytic site of the enzyme, the higher-order structure of which is still unknown.

Three genetic methods can easily be applied to the enzyme. Studies on the properties of altered enzymes from mutants isolated by random mutagenesis has indicated important (or essential) amino acid residues for catalysis and assembly. Random mutagenesis usually results in termination mutations and only limited numbers of amino acid substitutions when the numbers of essential residues are limited (Takeyama *et al.*, 1988; Miki *et al.*, 1986). Directed (or *in vitro*) mutagenesis of apparently conserved residues is more fruitful in such cases. This method also allows reevaluation of residues found to be important by analysis of random mutants or chemical modifications (affinity labeling). The isolations of pseudo-revertants of the mutant enzyme indicate that two amino acid residues (that from the original mutation and that from the pseudo-reversion) are functionally or structurally related. The isolations of pseudo-revertants of catalytically defective enzymes in the F_1 portion have started recently (Miki *et al.*, 1990; Iwamoto *et al.*, 1991). Pseudo-revertants of F_0 mutants defective in the *b* subunit have suggested close interactions of the membrane-embedded helix of the *b* subunit with the *a* and *c* subunits (Kumamoto and Simoni, 1986, 1987).

The sequence Gly-Gly-Ala-Gly-Val-Gly-Lys-Thr (residues 149–156 of the *E. coli* β subunit) in the F-type ATPase is one of the glycine-rich sequences found in many nucleotide-binding proteins including adenylate kinase and p21 *ras* protein (Walker *et al.*, 1984). In this minireview we discuss our combined approach "affinity labeling and genetic studies (random and directed mutants and their pseudo-revertants)" (Table I) to the glycine-rich sequence, and propose a model for the structure of the enzyme near

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Table I. Mutational Studies on the Glycine-Rich Sequence of the β Subunit of *E. coli* F-type ATPase[a]

Mutation	ATP synthase
βGly-149 → Ser	+
Ala	+
Cys	−
Thr	−
βGly-150 → Ser	+
βAla-151 → Val	−
Pro	+
βLys-155 → Ser	−
Ala	−
βThr-156 → Cys	−
Ala	−
βLys-155/βThr-156 → βThr-155/βLys-156	−

[a]Mutational studies on the glycine-rich sequence are summarized: mutations gave defective (−) or active (+) ATP synthase. For details, see text and references (Hsu *et al.*, 1987; Tekeyama *et al.*, 1990; Iwamoto *et al.*, 1991 and unpublished observations; Omote *et al.*, 1992).

the γ-phosphate moiety of ATP bound to the catalytic site (Fig. 1).

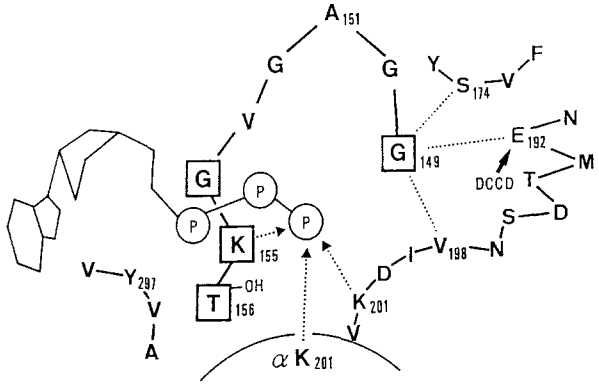

Fig. 1. Model of the glycine-rich sequence of the β subunit near the γ-phosphate moiety of ATP. A loop structure of the glycine-rich sequence of the β subunit was simulated from adenylate kinase (Takeyama *et al.*, 1990) and *ras* protein (Iwamoto *et al.*, 1991). Genetic studies indicated that βGly-149 is located near βSer-174, βGlu-192, and βVal-198 (Iwamoto *et al.*, 1991 and unpublished observations), and affinity labeling with AP_3-PL indicated that the γ phosphate moiety of ATP is near βLys-155, βLys-201, and αLys-201 (Tagaya *et al.*, 1988; Ida *et al.*, 1991). Experiment of Andrews *et al.* (1984) also suggests that βLys-155 is near βTyr-297. See text for details.

AFFINITY LABELING STUDIES

Affinity Labeling of the Glycine-Rich Sequence with Adenosine Triphosphopyridoxal (AP_3-PL)

Pyridoxal phosphate has been used to label lysine residues related to or in the catalytic sites of enzymes. Nucleotide di-, tri- or tetraphosphopyridoxals, a combination of a functional pyridoxal moiety with nucleotide, make pyridoxal phosphate a more specific affinity labeling reagent (Tagaya *et al.*, 1985). F_1-ATPase was inhibited by adenosine tri- (AP_3-PL) or tetra- (AP_4-PL) phosphopyridoxal, although it was less sensitive to pyridoxal phosphate and adenosine diphosphopyridoxal (AP_2-PL) (Noumi *et al.*, 1987): AP_3-PL inhibited both unisite and multisite catalyses, causing half maximal inactivation of multisite catalysis at 18 μM. More importantly, a titration curve of AP_3-PL concentration against loss of activity after binding indicated that the activity was completely lost by binding of 1 mole of AP_3-PL per mole of F_1. About 60% of the AP_3-PL bound to the α subunit and 40% to the β subunit, indicating that F_1-ATPase lost activity upon binding of the analogue to either the α or the β subunit.

Isolation and sequencing of peptides indicated that the αLys-201 and βLys-155 of the α and the β subunit residues, respectively, were labeled with AP_3-PL (Tagaya *et al.*, 1988). Addition of Mg^{2+} changed the labeling pattern, and AP_3-PL binding to either βLys-201 or βLys-155 inhibited the enzyme completely (Ida *et al.*, 1991). The F_1-ATPase used for labeling with Mg^{2+} had five bound nucieotides (three in noncatalytic sites, and two in catalytic sites), and one empty catalytic site (Hanada *et al.*, 1989). These results indicate that βLys-155 (in the glycine-rich sequence), βLys-201, and possibly αLys-201 are near the γ-phosphate moiety of ATP in the catalytic site.

Studies with 7-chloro-4-nitrobenzo-furazan suggested that βLys-155 is also close to βTyr-297 because the nitrobenzofurazan moiety bound to βTyr-297 was transferred to βLys-155 (Andrews *et al.*, 1984). Mutants of the α subunit (αLys-201 → Glu and αLys-201 deletion) were active in oxidative phosphorylation (Ida *et al.*, 1991). However, purified mutant F_1-ATPases showed lower multisite catalysis, although their unisite activities were essentially similar to that of the wild-type enzyme. These results suggest that αLys-201 or its vicinity is important for catalytic cooperativity. It is noteworthy that αLys-201 in the isolated α subunit was also labeled with adenosine diphosphopyridoxal (AP_2-PL) (Rao *et al.*, 1988). However, it is not known whether the site labeled in the isolated α subunit is exactly the same as that labeled in F_1.

AP_3-PL and GP_3-PL (guanosine triphosphopyridoxal) labeled corresponding Lys residues in the glycine-rich sequence of adenylate kinase (Tagaya *et al.*, 1987) and p21 *ras* protein Ohmi *et al.*, 1987), respectively. Thus nucleotide di-, tri-, or tetraphosphopyridoxal may be used to label Lys residues in glycine-rich sequences of other nucleotide-binding proteins. However, P-type ATPases (ion-translocating ATPases forming phospho-enzyme intermediates) such as H^+/K^+-ATPase were inhibited by pyridoxal phosphate as by AP_3-PL (Maeda *et al.*, 1988). The Lys residue (Lys-497) of H^+/K^+-ATPase labeled with pyridoxal phosphate (Tamura *et al.*, 1989) corresponded to the Lys residue of Na^+/K^+-ATPase labeled with AP_2-PL (Hinz and Kirley, 1990). Thus pyridoxal phosphate and adenosine di- or triphosphopyridoxal seem to bind to the same lysine residue of P-type ATPase.

GENETIC STUDIES

ATPases with Glycine-Rich Sequences from Other Proteins

The obvious and interesting question was whether the glycine-rich sequence of the β subunit could be replaced by those of other nucleotide-binding proteins (Takeyama *et al.*, 1990). We thought that it should be possible to replace the β sequence by those of other proteins, if these glycine-rich sequences share similar roles and structures. As expected, the enzyme with the *ras* protein sequence (Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser-Ala, residues 10–18) showed approximately 40% of the membrane ATPase activity of the wild type and was active in ATP synthesis. On the other hand, the enzyme with the adenylate kinase sequence (Gly-Gly-Pro-Gly-Ser-Gly-Lys-Gly-Thr, residues 15–23) had no activity.

These results were correlated with possible structural changes in the glycine-rich sequence. The structure of the sequence of the β subunit was simulated (Takeyama *et al.*, 1990: Iwamoto *et al.*, 1991) from the known three-dimensional structure of adenylate kinase (Sachsenheimer and Schulz, 1977; Dreusicke *et al.*, 1988) and p21 *ras* protein (Milburn *et al.*, 1990; Pai *et al.*, 1990). The predicted structure of the wild-type β subunit formed essentially the same loop structure as the sequences of adenylate kinase and *ras* protein (Takeyama *et al.*, 1990). However, there is a significant difference between the β subunit and adenylate kinase: adenylate kinase has a glycine insertion between the Lys and Thr residues, creating a difference in the position and projection of the side chain of the Thr residue. The Ser residue at the corresponding position of the *ras* sequence has a similar orientation to that of Thr-156 of the β subunit. Thus, the absence of activity of the adenylate kinase-like β mutant may be due to a change in orientation of the Thr sidechain. Consistent with these results, the β subunit with a glycine insertion between βLys-155 and βThr-156 had no activity (Takeyama *et al.*, 1990), whereas the βThr-156 → Ser mutant enzyme had essentially the same activity as the wild-type (Omote *et al.*, 1992).

βLys-155 → Ser or Ala, and βThr-156 → Cys or Ala Mutations Give F-Type ATPase with No Activity

Affinity labeling with AP_3-PL indicated that βLys-155 is near the γ phosphate moiety of ATP, as discussed above (Tagaya *et al.*, 1988; Ida *et al.*, 1991). Consistent with these results, the βLys-155 → Ser or Ala mutant enzyme showed no detectable multisite catalysis, and the ATP bindings (k_1) of these mutants in unisite catalysis were at least 30-fold lower than that of the wild-type enzyme (Omote *et al.*, 1992), indicating the βLys-155 is an essential residue for catalysis.

Results on the adenylate kinase type mutant (Takeyama *et al.*, 1990) suggested that βThr-156 itself may be essential. To evaluate this possibility, we introduced βThr-156 → Cys and βThr-156 → Ala substitutions (Iwamoto *et al.*, 1991) (Table 1). Neither mutant showed either multisite or unisite catalysis. In addition, we exchanged the positions of the two residues to βThr-155/βLys-156, which resulted in no enzyme activity (Omote *et al.*, 1992). These findings suggest that βThr-156 is essential for catalysis by the enzyme. βThr-156 could be replaced by Ser, because the βThr-156 → Ser mutant showed slightly higher ATPase activity. The replacement of the Thr residue by Ser in yeast ATPase also resulted in threefold higher activity (Mueller, 1989).

Altered Kinetics of the Enzyme with βAla-151 → Val or Pro Mutation

The enzyme with the βAla-151 → Val mutation showed low multisite and increased unisite catalysis (Hsu *et al.*, 1987). We also introduced a βAla-151 →

Pro mutation, because adenylate kinase (Gly-Gly-Pro-Gly-Ser-Gly-Lys-Gly-Thr, residues 15–23) has a Pro residue at the position corresponding to βAla-151 (Heil *et al.*, 1974). Interestingly, the rate of multisite catalysis with the (βPro-151) mutant enzyme was about twice that of the wild type (Takeyama *et al.*, 1990). The two mutations (βVal-151 and βPro-151) possibly had significant effects on the orientation of essential residues in the glycine-rich sequence such as βLys-155 and βThr-156 and changed the kinetics of the enzyme.

Amino Acid Residues Interacting Functionally with Residues in the Glycine-Rich Sequence

As the role of the glycine-rich sequence was defined from studies on affinity labeling and random and directed mutants, the residues structurally near or functionally interacting with the sequence could be identified by genetic procedures. Analysis of pseudo-revertants (suppressors) of mutants of the glycine-rich sequence could indicate these amino acid residues. If a first mutation is suppressed by a second mutation in the glycine-rich sequence, the residues replaced by the two mutations could interact functionally and/or be located close to each other. We found that the βSer-174 → Phe mutation was suppressed by the second mutation βGly-149 → Ser (Iwamoto *et al.*, 1991). The suppression was not specific for Ser substitution at position 149, because βGly-149 → Ala or Cys also suppressed the βPhe-174 mutant (Iwamoto *et al.*, unpublished observations). Analysis of mutations at position 149 showed that the Cys substitution alone resulted in a defective enzyme, whereas the βAla-149 and βSer-149 enzymes were active. On the other hand, βGly-149 → Thr or βGly-150 → Ser substitution did not suppress the βSer-174 → Phe mutation. Thus, the side chain volume of the amino acid residue at position 149 appears to be important for the suppression. These results strongly suggest that the βSer-174 and βGly-149 residues interact functionally with each other, and are possibly both located near the γ-phosphate moiety of ATP.

For further identification of the amino acid residues that interact functionally with βGly-149, pseudo-revertants for βCys-149 were isolated (Iwamoto *et al.*, unpublished observations). Mutations were introduced into the part of the β subunit gene corresponding to the amino acid residues between βLeu-162 and βArg-341, and the DNA fragment was ligated into a recombinant plasmid carrying the βCys-149 mutation. The plasmids were introduced into strain DK8, and strains capable of growing by oxidative phosphorylation were isolated. One of them harbored a recombinant plasmid carrying the entire *atp (unc)* operon with the βCys-149 mutation and a second mutation βVal-198 → Ala. The double mutant βCys-149 / βAla-198 was active in ATP synthesis and had similar membrane ATPase activity to the wild-type. βVal-198 is three residues upstream of βLys-201, which was shown to be near the γ phosphate moiety of ATP by affinity labeling with AP_3-PL in the presence of Mg^{2+} (Ida *et al.*, 1991), suggesting that βVal-198 is actually near βGly-149. Similarly, a double mutant βGly-149→Cys / βGlu-192→Ala was found to be active in ATP synthesis and had significant ATPase activity, indicating that βCys-149 is also near the βGlu-192 residue (DCCD[2] binding site, Yoshida *et al.*, 1982).

A MODEL OF THE CATALYTIC SITE NEAR THE γ-PHOSPHATE OF ATP

From these studies, we constructed a model for a part of the catalytic site near the γ phosphate moiety of ATP (Fig. 1). In this model the glycine-rich sequence forms a loop structure, and is located near the βSer-174, βGlu-192, βVal-198, βLys-201, and αLys-201 residues. βTyr-297 is also located near the glycine-rich sequence as judged by chemical modification (Andrews *et al.*, 1984). Our mutagenesis approach together with affinity labeling provided much information on the residues in (or near) the catalytic sites of F-type ATPase, the three-dimensional structure of which is as yet unknown. Determination of the three-dimensional structure should indicate whether the observed genetic interactions are due to direct structural interactions. The genetic method discussed above (random → directed → suppression) is tedious. However, at present it is the best method for analysis of complex enzymes such as F-type ATPase, and could also be used for studies of subunit–subunit interactions during catalysis.

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[2]DCCD—dicyclohexylcarbodiimide.

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