β Subunit Glu-185 of Escherichia coli H^+-ATPase (ATP Synthase) Is an Essential Residue for Cooperative Catalysis*

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The abbreviation used is: IAA, sodium iodoacetate.

Glu-β185 of the Escherichia coli H^+-ATPase (ATP synthase) β subunit was replaced by 19 different amino acid residues. The rates of multisite (steady state) catalysis of all the mutant membrane ATPases except Asp-β185 were less than 0.2% of the wild type one; the Asp-β185 enzyme exhibited 15% (purified) and 16% (membrane-bound) ATPase activity. The purified inactive Cys-β185 F₁₋₋₋ ATPase recovered substantial activity after treatment with iodoacetate in the presence of MgCl₂; maximal activity was obtained upon the introduction of about 3 mol of carboxymethyl residues/mole of F₁. The divalent cation dependences of the S-carboxymethyl-β185 and Asp-β185 ATPase activities were altered from that of the wild type. The Asp-β185, Cys-β185, S-carboxymethyl-β185, and Gln-β185 enzymes showed about 130, 60, 20, and 50% of the wild type unisite catalysis rates, respectively. The S-carboxymethyl-β185 and Asp-β185 enzymes showed altered divalent cation sensitivities, and the S-carboxymethyl-β185 enzyme showed no Mg^2+ inhibition. Unlike the wild type, the two mutant enzymes showed low sensitivities to azide, which stabilizes the enzyme MgADP complex. These results suggest that Glu-β185 may form a Mg^2+ binding site, and its carboxyl moiety is essential for catalytic cooperativity. Consistent with this model, the bovine glutamate residue corresponding to Glu-β185 is located close to the catalytic site in the higher order structure (Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621−628).

The H^+-ATPase (ATP synthase) of Escherichia coli synthesizes ATP similar to those of mitochondria or chloroplasts (see Refs. 1−4 for reviews). The catalytic site of the enzyme is in the β subunit of the membrane extrinsic F₁ sector. Studies on mutant enzymes indicated that Lys-β155 and Thr-β156 in the β subunit phosphate loop or conserved glycine-rich sequence (Gly-Gly-Ala-Gly-Val-Gly-Lys-Thr, residues 149−156; conserved residues underlined) and Glu-β181 and Arg-β182 in the conserved Gly-Glu-Arg sequence (residues 180−182) are essential catalytic residues (5−7). Affinity labeling with ATP analogues indicated that Lys-β155 bound the β and γ phosphate moiety of ATP (8). The crystal structure (9) of the bovine F₁ sector reported recently is essentially consistent with these results.

The purified F₁ (αβγδε or F₁-ATPase) hydrolyzes ATP through unisite (single site) or multisite (steady state) catalysis. The multisite rate is 10^5−10^6-fold faster than the unisite one due to the cooperativity of the multiple catalytic sites (10, 11). Conformational transmission for cooperativity may be initiated from a specific region(s) or residue(s) in the single catalytic site of the β subunit. Mutations near catalytic site residues often dramatically lower the multisite rate without changing unisite catalysis (6, 11, 12), possibly due to the defective conformational transmission between catalytic sites essential for the catalytic cooperativity. However, the role of a specific residue or region for the cooperativity has been questionable because all mutations so far introduced at a certain position did not always have the same effects on multisite catalysis. A typical example is the result of mutations at Gly-β149 of the phosphate loop; the Ala-β149 or Ser-β149 enzyme exhibited similar ATPase activity to the wild type, whereas the Cys-β149 enzyme had only 8% of the wild type ATPase activity (13), indicating that Gly-β149 is not an essential residue for conformational transmission.

In this study, we were interested in conserved Glu-β185, which is near essential catalytic residues (Glu-β181 and Arg-β182) described above and substituted it with 19 different residues. Surprisingly, all the mutants except Asp-β185 exhibited no multisite catalysis (less than 0.2% of the wild type activity); Asp-β185 had about 16% of the wild type membrane ATPase activity. Purified F₁₋₋₋ ATPases with Asp-β185, Gln-β185, and Cys-β185 residues showed unisite catalysis with rates of a similar order of magnitude to that of the wild type. The Cys-β185 enzyme showed substantial multisite catalysis upon chemical modification with sodium iodoacetate (IAA). These results clearly indicate that Glu-β185 is the first residue identified as being absolutely essential for multisite catalysis. The roles of Glu-β185 are discussed on the basis of the properties of the mutant enzymes.

EXPERIMENTAL PROCEDURES

E. coli and Growth Conditions—Strain DK8 (lacZ B-C, ilvTn10, thy) (14) lacking the unc operon was used as a host for recombinant plasmids. A rich medium (with or without 50 μg/ml ampicillin) supplemented with 50 μg/ml thymine and a minimal medium containing 50 μg/ml thymine, 2 μg/ml thiamine, 50 μg/ml isoleucine, 50 μg/ml valine, and 5 mM glucose (or 15 mM succinate) were used (15). Minimal medium with 0.5% glycerol was used for preparing membranes.

Construction of Recombinant Plasmids Carrying the unc Operon with Mutations at Position 185 of the β Subunit—Recombinant plasmids carrying mutations at position 185 of the β subunit were constructed using pUDS E709 (13). The following mutations were introduced by replacing the Xhol−Clal segment of pUDS E709 with the desired synthetic double stranded DNA: Gly (GTT), Ala (GCC), Ser (TCC), Thr (ACC), Asp (GAC), Asn (AAC), Glu (CAG), His (CAC), Lys (AAA), Arg (CGT), Cys (TGT), Tyr (TAC), Phe (TTC), Leu (CTG), Ile (ATG), Val

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(GTA), Met (ATG), Trp (TGG), and Pro (CCG). The Sad–EcoR11I segments of the resulting plasmids were transferred to pBWU14 carrying the entire unc operon (16).

Modification of Cys-185 F1-ATPase with IAA—Purified Cys-185 F1-ATPase was passed through a centrifuge column (Sephadex G-50, 0.4 cm × 6 cm) (17) equilibrated with 10 mM HEPES-NaOH, pH 8.0, to remove dithiothreitol and ATP included in the purified enzyme solution, and then incubated with 100 μM IAA in 50 mM HEPES-NaOH, pH 8.0, and 20 mM MgCl2, for 2 h at 30°C in the dark. The reaction was terminated by 100-fold dilution with a buffer (2 mM Tris-HCl, pH 8.0, 8 mM dithiothreitol, and 2 μM bovine serum albumin) or by removal of excess IAA using a centrifuge column.

Other Procedures—Membrane vesicles were prepared as described elsewhere (18) using 10 mM Tris-HCl buffer, pH 8.0, containing 140 mM KCl, 0.5 mM dithiothreitol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, and 5 μM pepstatin A. The Asp-185 and wild type F1-ATPases were purified as described previously (19). The Gin-185 and Cys-185 F1-ATPases were purified by the same procedure as for the wild type except that all column chromatography was carried out at room temperature. The enzyme contained about 0.5 mol of the α subunit/mg of protein. ATPase activities were assayed at 37°C in 20 mM Tris-HCl, pH 8.0, 4 mM ATP, and 2 mM MgCl2 (19) unless otherwise specified. One unit of the enzyme was defined as the amount hydrolyzing 1 μmol of ATP/min at 37°C under the above conditions. When indicated, varying concentrations of MgCl2 or CaCl2 were included in the reaction mixture.

Unisite catalysis was assayed using 0.25 μM [γ-32P]ATP and 0.5 or 10 mM MgSO4 at 25°C (20–23). The ATP binding rate (k_a) was measured as the decrease of ATP in the medium using hexokinase and glucose (5). The rate of ATP synthesis was assayed at 25°C by the published method (16). Protein concentration measurement (24) using bovine serum albumin as a standard and polyacylamide gel electrophoresis (25) were described previously.

Materials—Oligonucleotides were synthesized with Gene Assembler Plus (Pharmacia LKB). [α-32P]dCTP (3000 Ci/mmol) and N-ethyl-N-(1-13C)-ethyl maleimide (40 Ci/mmol) were purchased from Amersham Japan (Tokyo). [α-32P]ATP (10 Ci/mmol), [32P]dATP (3000 Ci/mmol), and [32P]dCTP (3000 Ci/mmol) were from ICN Biochemicals Corp. (Cleveland, OH), New England Biolabs Inc. (Beverly, MA), and Nippon Gene Co. (Toyama, Japan), respectively. Nippon Gene Co. (Toyama, Japan), Toyobo (Osaka, Japan), U. S. Biochemical Corp. (Cleveland, OH), or New England Biolabs Inc. (Beverly, MA). Other reagents used were of the highest grade commercially available.

RESULTS

TABLE 1

| Residue at position 185 | Growth on succinate | Membrane ATPase | F1-F2 assembly |
|------------------------|---------------------|-----------------|----------------|
| Glu (wild type)        | +   | 100   | ++    |
| Asp                    | +   | 16.4  | ++    |
| Gin, Ser, Lys, Ala, Gly, Leu, Met, Phe, Trp, or Pro | -   | <0.2  | ++    |
| Tyr, Asn, Thr, Arg, His, Cys, Val, or Ile        | --  | <0.2  | +     |

Properties of 19 Mutants at Position 185 (Glu, wild type) of β Subunit—Systematic mutagenesis between Thr-185 and Lys-201 of the β subunit indicated that Glu-181 and Arg-182 are essential for catalysis (6). We were interested in the conserved Glu-185 residue located near these residues and replaced it with 19 different residues including Glu. All the mutants except Asp-185 could not grow on succinate through oxidative phosphorylation, although they exhibited substantial F1F2 assemblies in membranes (Table I). The Asp-185 mutant showed essentially the same growth yield as the wild type. Eight mutants (Tyr-185, Asn-185, Thr-185, Arg-185, His-185, Cys-185, Val-185, and Ile-185) exhibited about 50% of the wild type assembly, whereas the others exhibited essentially similar assembly to that in the case of the wild type. The low degrees of assemblies in the eight mutants may suggest that Glu-185 is located in the critical region for interaction of the β subunit with other subunit(s). The importance of Glu-185 for subunit assembly was suggested previously by the fact that the isolated Glu-185 and Lys-185 F1 and β subunits could not form an α2β2γ complex in vitro (26).

Asp-185 exhibited about 16% of the wild type membrane ATPase activity, whereas other mutants exhibited no membrane ATPase activity (less than 0.2% of wild type multisite catalysis). Membrane ATPase of the Cys-185 mutant became detectable after incubation with IAA but not with the same concentration of iodoacetamide; activity of about 0.05 units/mg protein became detectable after incubation of Cys-185 membranes with 100 μM IAA in 50 mM Tris-HCl, pH 8.0, at room temperature for 10 min (ATPase activity of Cys-185 F1-ATPase without IAA treatment, about 0.01–0.02 units/mg). This result suggests that the βs-carboxymethylated enzyme has activity. Detailed studies of the IAA effects were then carried out below using purified Cys-185 F1-ATPase. Consistent with the low membrane ATPase activity and negative growth by oxidative phosphorylation, mutant membranes (Gln-185 or Cys-185) did not show significant ATP synthesis (Table II, right column). Membranes treated with IAA also did not show ATP synthesis, because the mutant membranes lost respiration-driven proton transport after IAA treatment (data not shown). A similar IAA effect was observed for wild type membranes.

Properties of Purified Mutant Enzymes—Three mutant F1-ATPases (Asp-185, Gin-185, and Cys-185) were purified using a procedure developed for the wild type; they behaved similarly during column chromatographies and showed essentially the same recoveries as that of the wild type (about 50% from the EDTA extract). The Gin-185 and Cys-185 enzyme showed no multisite catalysis with ATP (∼0.1% of the wild type level), ITP, or GTP as a substrate (with MgCl2 or CaCl2 as a divalent cation). Both enzymes did not show ATPase activity after incubation with pyruvate kinase and phosphoenolpyruvate to remove endogenous exchangeable ADP (Ref. 27 and data not shown), suggesting that the enzymes are not in the highly inhibited state with Mg-ADP. On the other hand, the Asp-185 enzyme showed about 15% of the wild type rate, similar to the membrane enzyme.

The mutant enzymes showed unisite catalysis with initial rates of about 50 (Gln-185), 60 (Cys-185), and 130% (Asp-185) of that of the wild type, and Asp-185 F1-ATPase exhibited a k_z value (rate of ATP binding) of a similar order of magnitude as that of the wild type (Table I). The k_z values for Gln-185 and Cys-185 were slightly lower than that of the wild type. The wild type and all the mutant enzymes except Cys-185 F1-ATPase showed cold chase in unisite catalysis, consistent with the partial release of the β subunit from F1 during purification (23). These results clearly indicate that the major defect of the mutant enzymes is not in the catalytic reaction itself but in the catalytic cooperativity required for multisite catalysis.

Activation of the Purified Cys-185 F1-ATPase with IAA—Multisite catalysis of the purified Cys-185 F1-ATPase was very low but became detectable when it was incubated with IAA (Table I). The activation was dependent on the IAA concentration; about 2 μmol/mg ATPase activity/mg protein
became detectable after incubation with 100 μM IAA (Fig. 1a, closed circles). The activity increased dramatically with the addition of MgCl₂, maximal activity (10 μmol/mg-min protein) was obtained with 100 μM IAA and 20 mM MgCl₂ (Fig. 1a, open circles; Fig. 1b). CaCl₂ had less effect on the activation by IAA; the maximal activity obtained was about 4 μmol/mg-min protein (Fig. 1b, diamonds). These results indicate that the Cys-β185 residue became more reactive to IAA upon the addition of MgCl₂. The maximal activity obtained corresponds about 30% of that of the wild type. Similar activation was not observed with other sulfhydryl reagents (1 mM), such as iodoacetamide, diithiobis(2-nitrobenzoic acid), 4-chloro-7-sulfobenzofurazan, and N-ethylmaleimide, indicating that the carboxyl moiety introduced at position 185 after incubation with IAA is essential for enzyme activation. The initial rate and kₘ of unisite catalysis by the S-carboxymethyl-β185 enzyme were about ¼ and ¼ of those of the wild type enzyme, respectively (Table II). These results clearly indicate that the increased multisite catalysis described above was not due to the increased rate of unisite catalysis.

**Table II**

| Enzyme residue at position β185 | Multisite catalysis | Unisite catalysis | Membrane ATP synthesis |
|---------------------------------|---------------------|------------------|------------------------|
|                                 | sec⁻¹ | mmol/mg/min | 10⁶ sec⁻¹ M⁻¹ | nmol/mg/min |
| Glu (wild type)                 | 148 (50.8) | 1.2 | 9.7 | 90 |
| Asp                             | 22.4 | 1.6 | 13 | 22 |
| Gln                             | 0.05 | 0.55 | 5.9 | <0.1 |
| Cys                             | <0.05 | 0.73 | 3.2 | <0.1 |
| S-Carboxymethyl                 | 1.6 (6.2) | 0.27 | 0.92 | <0.1 |

**DISCUSSION**

Extensive mutagenesis studies on F₁-ATPase showed that the Lys-β155 and Thr-β156 residues of the phosphate loop (5) and Glu-β181 (6, 7) and Arg-β182 (6) of the conserved Gly-Glu-Arg (positions 180-182) sequence are essential residues for unisite and multisite catalysis. Thus, the roles of other residues near the phosphate loop and the Gly-Glu-Arg sequence are of interest. We were interested in the Glu-β185 residue, which
conserved in all the β subunits so far sequenced (57 different species; SWISS PROT Release 30). It was surprising to find that all the mutants except Asp-β185 were unable to grow by oxidative phosphorylation and exhibited no functional multisite catalysis. The purified Gln-β185 and Cys-β185 F₁-ATPases also exhibited no multisite catalysis. Cross and co-workers (27) showed recently that E. coli F₁-ATPase, similar to chloroplast or mitochondrial F₁ (30), is inhibited by the catalytic site-bound MgADP. They proposed that the effect of MgADP should be considered before kinetic results are interpreted. However, we think that the possibility of highly increased MgADP inhibition of mutant enzymes is low because phosphoenolpyruvate and pyruvate kinase (treatment to release MgADP) did not increase the activities of the Gln-β185 and Cys-β185 F₁-ATPases. Furthermore, the S-carboxymethyl-β185 and Asp-β185 enzymes were not inhibited by Mg²⁺, as discussed below.

Despite the absence of multisite catalysis, the purified mutant F₁-ATPases (Gln-β185 and Cys-β185) retained substantial unisite catalysis. Furthermore, multisite catalysis of the Cys-β185 enzyme was recovered on the introduction of a carboxymethyl group after treatment with IAA, whereas the same treatment did not increase the unisite catalysis of the enzyme. Taken together with the observation of Asp-β185 mutant, these results indicate that the carboxyl moiety at position 185 is required for catalytic cooperativity. It is noteworthy that Glu-β185 is the first residue found to be essential for multisite catalysis. Similar residues were not identified previously because multisite catalysis was lost to varying degrees depending on the residues substituted (16, 31).

MgCl₂ had a dramatic effect on the activation of Cys-β185 F₁-ATPase with IAA; ATPase activity obtained with MgCl₂ was about 5-fold higher than that on incubation without it. About 3 and 2 mol of S-carboxymethyl residues were incorporated into the mutant enzyme, respectively, on incubation with and without MgCl₂, respectively. Thus, all three Cys-β185 residues bound carboxymethyl moieties in the presence of Mg²⁺ and became fully active, consistent with the requirement of three active β subunits for multisite activity (11).

The S-carboxymethyl-β185 and Asp-β185 enzymes had...
two mutant enzymes required more MgCl₂ for maximal multi-cation dependences different from those of the wild type: the interesting properties. Their ATPase activities showed divalent cation requirements through Mg²⁺ of control (without LiCl). The control rates were given in the legend to structure of bovine F₁-ATPase (9). Thus, we propose that the Mg²⁺ binding. In this regard, Weber and co-workers reported that the cooperativity for ATP binding is dependent on Mg²⁺ (32).

In contrast to the strong inhibition of the wild type enzyme by MgCl₂ at higher than 3 mM (about 60% inhibition with 5 mM MgCl₂), excess MgCl₂ did not inhibit the multisite catalysis of S-carboxymethyl-βS₁₈₅ and only slightly inhibited the Asp-βS₁₈₅ enzyme (about 10% inhibition with 10 mM MgCl₂). The Mg²⁺ inhibition of the ATPase activity of the wild type enzyme was shown to be due to the MgADP binding to the catalytic site (27). Similar to wild type enzyme, S-carboxymethyl-βS₁₈₅ and Asp-βS₁₈₅ enzyme retained about five bound nucleotides detected after passing through a centrifuge column (data not shown). Thus, the low Mg²⁺ inhibition of the mutant enzymes suggests that the affinity of the Mg²⁺ ion to the catalytic site bound ADP was lower in the mutant than in the wild type. In addition, the azide sensitivities of the S-carboxymethyl-βS₁₈₅ and Asp-βS₁₈₅ enzymes were decreased by more than 2 orders of magnitude. Azide inhibits Fₐ-ATPase by stabilizing the enzyme-MgADP complex (27, 30), suggesting that the low azide sensitivity of Asp-βS₁₈₅ or S-carboxymethyl-βS₁₈₅ is because the mutant enzyme-MgADP complex is not stabilized by azide. Previously, we reported that azide did not inhibit multisite catalysis (22). Thus, azide may change the environment around the Mg²⁺ ion binding site including the Glu-βS₁₈₅ residue, resulting in strong inhibition of the catalytic cooperativity through stabilization of the enzyme-MgADP complex.

Fig. 4. Sodium azide sensitivities of the S-carboxymethyl-βS₁₈₅ and Asp-βS₁₈₅ enzymes. The mutant (S-carboxymethyl-βS₁₈₅ (squares) and Asp-βS₁₈₅ (triangles)) and wild type (circles) Fₐ-ATPases were assayed with varying concentrations of NaN₃ in the presence of 4 mM ATP and 10 mM MgCl₂. The results are expressed as relative rates of percentage of control (without azide). The control values for the mutant and wild type enzymes were S-carboxymethyl-βS₁₈₅, 12.6; Asp-βS₁₈₅, 26.4; and wild type, 32.4 μmol/mg/min.

Fig. 5. Effects of LiCl on the S-carboxymethyl-βS₁₈₅ and Asp-βS₁₈₅ enzymes. The mutant (S-carboxymethyl-βS₁₈₅ (triangles) and Asp-βS₁₈₅ (squares)) and wild type (circles) Fₐ-ATPases were assayed with varying concentrations of LiCl in the presence of 4 mM ATP and 10 mM MgCl₂. The results are expressed as relative rates of percentage of control (without LiCl). The control rates were given in the legend to Fig. 4.

interesting properties. Their ATPase activities showed divalent cation dependences different from those of the wild type: the two mutant enzymes required more MgCl₂ for maximal multisite catalysis than the wild type and exhibited very low CaCl²⁻ dependent activity. Interestingly, S-carboxymethyl-βS₁₈₅ enzyme activity is accelerated by excess MgCl₂ (4 mM ATP and 10 mM MgCl₂), suggesting the importance of free Mg²⁺ ion. On the other hand, the divalent cation requirements of the mutant enzymes for multisite catalysis were similar to those of the wild type (data not shown). Thus, a change in the side chain length of the carboxyl moiety (Asp, Glu, and S-carboxymethyl) at position 185 affected the divalent cation requirement for multisite catalysis. These results suggest that the carboxyl group of the Glu-βS₁₈₅ residue may be close to Mg²⁺ at the catalytic site or forming the Mg²⁺ binding site. The bovine glutamate (position 192) residue corresponding to E. coli Glu-βS₁₈₅ is actually located in the catalytic site close to the Mg²⁺ ion in the x-ray structure of bovine Fₐ-ATPase (9). Thus, we propose that the Glu-βS₁₈₅ residue contributes to the catalytic cooperativity through Mg²⁺ binding. In this regard, Weber and co-workers

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