Identification of α-subunit Lys201 and β-subunit Lys115 at the ATP-binding sites inEscherichia coli F1-ATPase

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Identification of α-subunit Lys^{201} and β-subunit Lys^{155} at the ATP-binding sites in *Escherichia coli* F_1-ATPase

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Binding of about 1 mol of adenosine triphosphopyridoxal to *Escherichia coli* F_1-ATPase resulted in the nearly complete inactivation of the enzyme [(1987) J. Biol. Chem. 262, 7686–7692]. About two thirds of the label was bound to the α-subunit, and the rest to the β-subunit. The present study revealed that Lys^{201} in the α-subunit and Lys^{155} in the glycine-rich region of the β-subunit are the major sites labeled with this reagent. Thus, these two residues might be located close to the γ-phosphate of the bound ATP.

F_1-ATPase; Nucleotide-binding site; Affinity label; Adenosine polyphosphopyridoxal; Glycine-rich region

1. INTRODUCTION

Escherichia coli H^+-ATPase catalyzes ATP synthesis from ADP and inorganic orthophosphate coupled with an electrochemical gradient of protons. The catalytic portion of the enzyme, F_1, has a structure of $\alpha_3\beta_3\gamma\delta\epsilon$ and contains six nucleotide-binding sites; three nonexchangeable (noncatalytic) and three exchangeable (catalytic) sites [1–3]. Several lines of evidence indicate that both α- and β-subunits contain nucleotide-binding sites [4–6]. A photoaffinity cross-linking study showed the presence of nucleotide-binding sites at the interface between the α- and β-subunits [7]. Cross et al. [8] suggested that the catalytic and noncatalytic sites are located in the β-subunit and at the interface between the α- and β-subunits, respectively.

We previously showed that the binding of about 1 mol of AP_3-PL to 1 mol of *E. coli* F_1-ATPase caused nearly complete inactivation of the enzyme [9]. About two-thirds of AP_3-PL was bound to the α-subunit, and the rest to the β-subunit. The kinetic studies of inactivation provided evidence that the reagent binds to the catalytic sites of the enzyme [9]. The results of the present study demonstrate that Lys^{201} in the α-subunit and Lys^{155} in the β-subunit are predominantly labeled with AP_3-PL. The latter residue is located in the glycine-rich region which is often observed in nucleotide-binding proteins [10,11].

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Abbreviations: AP_3-PL, adenosine triphosphopyridoxal; FSBA, 5′-*p*-fluorosulfonylbenzoyl adenosine; FSBI, 5′-*p*-fluorosulfonylbenzoyl inosine; HPLC, high performance liquid chromatography

2. EXPERIMENTAL

AP_3-PL was synthesized according to the method of Tagaya and Fukui [12]. Asahipak ODP (6 × 150 mm) and Synchropak RP-P (4.1 × 250 mm) were obtained from Asahi Kasei Co. and M&S Instruments, respectively. Lysyl endopeptidase was obtained from Wako Chemical. Other proteases used in this study were purchased from Worthington.

ATP-hydrolyzing activity was assayed at 37°C in 50 mM Tris-HCl (pH 8.0) containing 4.0 mM ATP, 2.0 mM $MgCl_2$, and an appropriate amount of the enzyme. ITP-hydrolyzing activity was measured using 2.0 mM ITP and 1.0 mM $MgCl_2$ under similar conditions.

AP_3-PL-labeled peptides were purified as follows. About 90 mg of *E. coli* F_1-ATPase (70 ml) which had been passed through a column (1.6 × 90 cm) of Sephadex G-50 (coarse) equilibrated with 50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 8.0)

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was incubated with 0.1 mM AP_3-PL at 25°C for 40 min. To this solution was added 1.4 ml of a freshly prepared 0.1 M $NaBH_4$ solution, and the mixture was left to stand for 20 min. The modified enzyme was dialyzed against 0.5 M Tris-HCl (pH 8.6) containing 13 mM EDTA, and carboxymethylated as described by Reimann et al. [13]. The carboxymethylated protein was dialyzed against water, and lyophilized. The lyophilized materials were dissolved in 2 ml of 0.1 M Tris-HCl (pH 9.0) containing 8 M urea, and then diluted with 2 ml of 0.1 M Tris-HCl (pH 9.0). The protein was digested at 37°C for 6 h with lysyl endopeptidase in the ratio of protein to protease of 100:1. The digest was applied to a column (2.3 × 170 cm) of Sephadex G-50 (super fine) equilibrated with 0.2 M NH_4HCO_3-NH_4OH (pH 9.0), and 3.0-ml fractions were collected. Fractions 112–123 and 147–157 were separately pooled, and lyophilized.

Fractions 112–123 (peak II): the lyophilized fragments dissolved in 1 ml of 0.1 M Tris-HCl (pH 8.0) were incubated with 20 μg of *Staphylococcus* V8 protease at 37°C overnight. The digest was applied to a column (2.8 × 90 cm) of Bio-Gel P-6 equilibrated with 0.2 M NH_4HCO_3-NH_4OH (pH 9.0), and 2.4-ml fractions were collected. The fluorescent material eluted as a single peak was pooled, lyophilized, and dissolved in 1 ml of 1 mM NaOH. A portion of the solution was subjected to HPLC with Asahipak ODP at a flow rate of 0.75 ml/min. The solvents used were (A) 20 mM NH_4OH-CH_3COOH (pH 9.0) and (B) 20 mM NH_4OH-CH_3COOH (pH 9.0) containing 80% acetonitrile. Gradients were run as follows: time 0–80 min, a linear gradient of (B) from O–40%; 85–90 min, linear gradient of (B) from 40–90%.

Fractions 147–157 (peak IV): the lyophilized fragments dissolved in 0.1 M NH_4HCO_3 were incubated with 25 μg of trypsin at 37°C for 6 h. The digest was applied to a column (2.8 × 90 cm) of Bio-Gel P-6 equilibrated with 0.1 M NH_4HCO_3-NH_4OH (pH 9.0), and 2.2-ml fractions were collected. The fluorescent material eluted as a single peak was pooled and lyophilized. The lyophilized material was dissolved in 1 ml of 0.1 M NH_4HCO_3, and a portion of the solution (0.1 ml) was further digested with 25 μg of chymotrypsin at 37°C for 10 h. The digest was applied to HPLC with Synchropak RP-P at a flow rate of 0.75 ml/min. The solvents used were (A) 0.1% trifluoroacetic acid containing 5% acetonitrile and (B) 0.1% trifluoroacetic acid containing 90% acetonitrile. Gradients were run as follows: time 0–10 min, buffer (B) 0%; time 10–70 min, a linear gradient of buffer (B) from 0–30%; time 70–75 min, a linear gradient of buffer (B) from 30–90%. The labeled peptides were purified by rechromatography with the same column under the following conditions. The solvents were (A) 10 mM CH_3COONH_4 and (B) 10 mM CH_3COONH_4 containing 90% acetonitrile. Gradients were run as follows: time 0–40 min, a linear gradient of buffer (B) from 0–15%; time 40–45 min, a linear gradient of buffer (B) from 15–90%.

3. RESULTS

Previous results suggested that AP_3-PL binds to the catalytic sites of F_1-ATPase [9]. A further criterion for the binding to the catalytic sites is the same sensitivity of ATP- and ITP-hydrolyzing activities of the enzyme to the reagent (cf. 14,15]. When ATP- and ITP-hydrolyzing activities were measured after modification of the F_1-ATPase with various concentrations of AP_3-PL, both activities were inhibited to the same degree (fig.1), supporting the binding of AP_3-PL to the catalytic sites.

AP_3-PL-labeled *E. coli* F_1-ATPase was digested with lysyl endopeptidase, and a mixture of fragments were applied to a Sephadex G-50 column (fig.2). Fluorescence due to the pyridoxyl moiety of the label was eluted as four peaks. The first fluorescent peak (peak I) was eluted at a position corresponding to the void volume of the col-

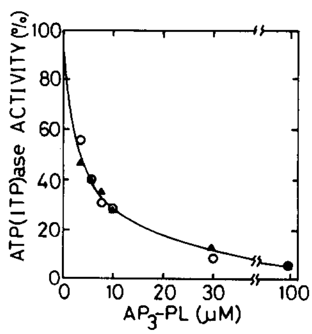

Fig.1. Comparison of the inactivation of the ATP- and ITP-hydrolyzing activities of F_1-ATPase by AP_3-PL. *E. coli* F_1-ATPase was incubated with AP_3-PL at various concentrations (4–100 μM) at 25°C in 50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 8.0). After 30 min, $NaBH_4$ was added to a final concentration of 2 mM to fix the inhibitor, and then ATP- (○) and ITP- (▲) hydrolyzing activities were measured.

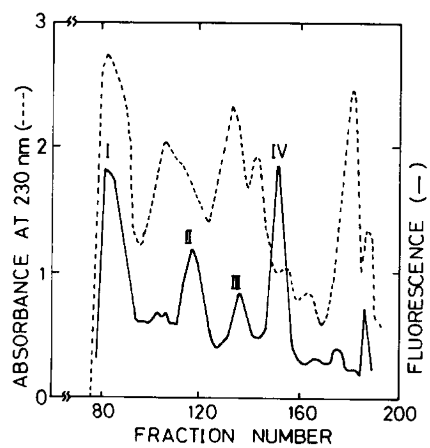

Fig.2. Gel filtration of AP_3-PL-labeled F_1-ATPase after digestion with lysyl endopeptidase. Details are described in section 2.

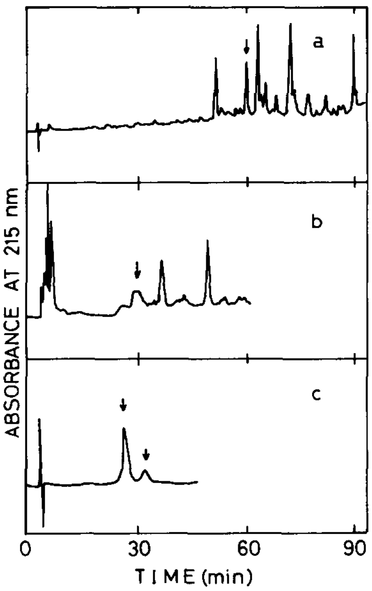

Fig.3. HPLC of AP_3-PL-labeled peptides. The elution patterns of *Staphylococcus* V8 protease-digested peptides of peak II (a) and trypsin-digested peptides of peak IV (b). Rechromatography of the fluorescent material in the trypsin-digested peptides of peak IV (c). Arrows indicate the peaks with fluorescence. Details are described in section 2.

umn, suggesting that peak I is the undigested material. The amount of fluorescence in peak III was much smaller than those of peaks II and IV. In the preliminary experiment using a small amount of F_1-ATPase labeled with $[^3H]AP_3$-PL, only two radioactive peaks corresponding to peak II and IV were observed (data not shown). Therefore, peak III may be an incompletely cleaved peptide(s) or nonspecifically labeled peptide(s). We did not further analyze peaks I and III.

The peptide mixture in peak II was digested with *Staphylococcus* V8 protease, and the labeled peptide was purified by a combination of Bio-Gel P-6 gel chromatography and HPLC under alkaline conditions. In each purification step, essentially only one fluorescent peak was observed. As shown in fig.3a, a fluorescent peptide was eluted at 61 min on HPLC with 45% yield. Sequence analysis (table 1) showed that this peptide corresponds to a segment from Val^{145} to Glu^{161} in the β-subunit of F_1-ATpase [16]. No phenylthiohydantoin derivative of amino acid was detectable at cycle 11. We surmise from the known sequence of the β-subunit [16] that the 11th amino acid is a labeled lysine (Lys^{155}).

Table 1

Amino acid sequences of AP_3-PL-labeled peptides

Peak II			Peak IV					
			Major			Minor		
Cycle no.	Amino acid	Yield (pmol)	Cycle no.	Amino acid	Yield (pmol)	Cycle no.	Amino acid	Yield (pmol)
1	Val	870	1	Val	110	1	Val	28
2	Gly	410	2	Ala	100	2	Ala	30
3	Leu	280	3	Ile	75	3	Ile	36
4	Phe	210	4	Gly	71	4	Gly	31
5	Gly	220	5	Gln	53	5	Gln	39
6	Gly	200	6	N.I.[a]		6	N.I.	
7	Ala	230	7	Ala	60	7	Ala	23
8	Gly	160	8	Ser	–	8	Ser	–
9	Val	150	9	N.I.				
10	Gly	130	10	Ile	23			
11	N.I.							
12	Thr	–						
13	Val	81						
14	Asn	76						
15	Met	89						
16	Met	94						
17	Glu	8						

[a] Not identified

The peptide mixture in peak IV was digested with trypsin, and only one fluorescent peak was observed on Bio-Gel P-6 chromatography. The fluorescent fractions were pooled, lyophilized, and then digested with chymotrypsin. Chymotryptic fragments were applied to HPLC and eluted with trifluoroacetic acid. Fluorescent materials were eluted at 30 min as a broad peak with 70% recovery (fig.3b). The peak material was applied to the same column and eluted with ammonium acetate (fig.3c). Two fluorescent peptides were eluted at 26 and 32 min in the fluorescence ratio of approx. 3:1. Sequence analyses (table 1) showed that the structures of the two peptides are the same and correspond to a segment from Val^{196} to Ile^{205} in the α subunit [16]. No phenylthiohydantoin derivative of amino acid was detectable at cycle 6. We surmise that the 6th amino acid is a labeled lysine (Lys^{201}).

4. DISCUSSION

The results of the present study demonstrate that Lys^{201} in the α-subunit and Lys^{155} in the β-subunit are the major sites labeled with AP_3-PL. These residues are completely conserved in all the known sequences of F_1-ATPases except that Lys^{201} in the α-subunit is conservatively substituted by arginyl residue in the wheat chloroplast enzyme [17]. Furthermore, the regions in their vicinities, especially around Lys^{155} in the β-subunit, are highly conserved, consistent with the idea that these residues play important roles in enzyme catalysis.

Bullough and Allison [14,15] showed that the complete inactivation of bovine F_1-ATPase is accompanied by the incorporation of 1 mol of FSBI per mol of enzyme, whereas the binding of 3 mol of FSBA per mol of enzyme is necessary for the complete inactivation. Since the noncatalytic sites are highly specific for adenine nucleotides [18], they suggested that FSBI and FSBA bind to the catalytic and noncatalytic sites, respectively. This suggestion was confirmed by the finding that 1 mol of 2-azido ATP binds to the catalytic sites per mol of F_1 and modifies the same tyrosyl residue as FSBI [8]. Thus, the binding of 1 mol of an adenine nucleotide analogue to the catalytic sites might be enough for the complete inactivation. Our previous study revealed that binding of 1 mol of AP_3-PL per mol of enzyme resulted in nearly complete inactivation [9]. In accordance with the binding of FSBI to the catalytic sites, both ATP- and ITP-hydrolyzing activities decreased at the same rate [15]. Similarly, both activities showed the same sensitivities to AP_3-PL (fig.1). Based on the present findings and the detailed kinetic analysis [9], we conclude that AP_3-PL binds to the catalytic sites of the enzyme. Kironde and Cross [19] discussed the location of the catalytic and noncatalytic sites in F_1-ATPase, and proposed several models. However, the evidence accumulated is still not enough to conclude exclusively which model is correct. Since the possibility that both α- and β-subunits have independent catalytic sites is unlikely, our results are consistent with the model that the catalytic sites are located at the interface between the α- and β- subunits [7].

4-Chloro-7-nitrobenzofurazan also modified Lys^{155} in the β-subunit [20], which is within the conserved sequence [Gly-X-X-X-X-Gly-Lys-Ser(Thr)] of nucleotide-binding proteins such as adenylate kinase [10,11]. The analysis of the mutant enzyme [21] and the results of site-directed mutagenesis studies [22,23] suggested the importance of the conserved sequence and Lys^{155} for enzyme activity. It is generally assumed that the lysyl residue in this region is located close to the γ-phosphate of the bound ATP on analogy of adenylate kinase, of which three-dimensional structure was resolved [24]. However, the exact location of the nucleotide-binding sites in adenylate kinase could not be concluded from physical studies [11,25]. We have recently demonstrated that AP_3-PL binds to Lys^{21}, located in the conserved sequence, and suggested that the ϵ-amino group of Lys^{21} is in the vicinity of the γ-phosphate of the bound ATP [26,27]. The present study provided the evidence that Lys^{155} in the β-subunit of F_1-ATPase is also located close to the γ-phosphate of the bound ATP.

In addition to Lys^{155} in the β-subunit, Lys^{201} in the α-subunit might also be located close to the γ-phosphate of the bound ATP. Since both subunits show considerable sequence homology [16,17], it would be expected that their secondary and tertiary structures are similar. However, AP_3-PL did not bind to Lys^{175} in the α-subunit, a lysyl residue corresponding to Lys^{155} in the β-subunit, suggesting that the residues involved in the binding of nucleotides are different from each other in the two subunits. Based on the prediction of the secondary structure, Lys^{155} in the β-subunit seems to be located in a loop between an α-helix and a β-structure [28]. Lys^{201} in the α-subunit is also expected to be located at a turn between an α-helix and a β-structure [29]. The locations of the two lysyl residues are reasonable because adenine nucleotides generally bind to the edge of α/β folding ('Rossmann fold', review [30]). Yamamoto et al. [31] have recently shown that AP_3-PL binds to Lys^{684} in sarcoplasmic reticulum Ca^{2+}-ATPase. However, no sequence homology was observed in the AP_3-PL-labeled region between F_1-ATPase and Ca^{2+}-ATPase.

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REFERENCES

[1] Senior, A.E. and Wise, J.G. (1983) J. Membr. Biol. 73, 105–124.
[2] Harris, D.A. (1978) Biochim. Biophys. Acta 463, 245–273.
[3] Cross, R.L. and Nalin, C.M. (1982) J. Biol. Chem. 257, 2874–2881.
[4] Vignais, P.V. and Lunardi, J. (1985) Annu. Rev. Biochem. 54, 977–1014.
[5] Dunn, S.D. and Futai, M. (1980) J. Biol. Chem. 255, 113–118.
[6] Gromet-Elhanan, Z. and Khananshvili, D. (1984) Biochemistry 23, 1022–1028.
[7] Schafer, H.-J. and Dose, K. (1984) J. Biol. Chem. 259, 15301–15306.
[8] Cross, R.L., Cunningham, D., Miller, C.G., Xue, Z., Zhou, J.-M. and Boyer, P.D. (1987) Proc. Natl. Acad. Sci. USA 84, 5715–5719.
[9] Noumi, T., Tagaya, M., Miki-Takeda, K., Maeda, M., Fukui, T. and Futai, M. (1987) J. Biol. Chem. 262, 7686–7692.
[10] Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–951.
[11] Fry, D.C., Kuby, S.A. and Mildvan, A.S. (1986) Proc. Natl. Acad. Sci. USA 83, 907–911.
[12] Tagaya, M. and Fukui, T. (1986) Biochemistry 25, 2958–2964.
[13] Reimann, E.M., Titani, K., Ericsson, L.H., Wade, R.D., Fischer, E.H. and Walsh, K.A. (1984) Biochemistry 23, 4185–4192.
[14] Bullough, D.A. and Allison, W.S. (1986) J. Biol. Chem. 261, 5722–5730.
[15] Bullough, D.A. and Allison, W.S. (1986) J. Biol. Chem. 261, 14171–14177.
[16] Kanazawa, H. and Futai, M. (1982) Ann. NY Acad. Sci. 402, 45–64.
[17] Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M. and Tybulewicz, V.L.J. (1985) J. Mol. Biol. 184, 677–701.
[18] Harris, D.A., Gomez-Fernandez, J.C., Klungsoyr, L. and Radda, G.K. (1978) Biochim. Biophys. Acta 504, 364–383.
[19] Kironde, F.A.S. and Cross, R.L. (1987) J. Biol. Chem. 262, 3488–3495.
[20] Andrews, W.W., Hill, F.C. and Allison, W.S. (1984) J. Biol. Chem. 259, 14378–14382.
[21] Hsu, S.-Y., Noumi, T., Takeyama, M., Maeda, M., Ishibashi, S. and Futai, M. (1987) FEBS Lett. 218, 222–226.
[22] Parsonage, D., Wilke-Mounts, S. and Senior, A.E. (1987) J. Biol. Chem. 262, 8022–8026.
[23] Yoshida, M., Ohta, S., Hisabori, T. and Kagawa, Y. (1988) Biochim. Biophys. Acta, in press.
[24] Schulz, G.E., Elzinga, M., Marx, F. and Schirmer, R.H. (1974) Nature 250, 120–123.
[25] Pai, E.F., Sachsenheimer, W., Schirmer, R.H. and Schulz, G.E. (1977) J. Mol. Biol. 114, 37–45.
[26] Tagaya, M., Yagami, T. and Fukui, T. (1987) J. Biol. Chem. 262, 8257–8261.
[27] Yagami, T., Tagaya, M. and Fukui, T. (1988) FEBS Lett. 229, 261–264.
[28] Parsonage, D., Duncan, T.M., Wilke-Mounts, S., Kironde, F.A.S., Hatch, L. and Senior, A.E. (1987) J. Biol. Chem. 262, 6301–6307.
[29] Maggio, M.B., Pagan, J., Parsonage, D.P., Hatch, L. and Senior, A.E. (1987) J. Biol. Chem. 262, 8981–8984.
[30] Holbrook, J.J., Liljas, A., Steindel, S.J. and Rossmann, M.G. (1975) in: The Enzymes (Boyer, P.D. ed.) 3rd edn, vol. XI, pp. 191–292, Academic Press, Orlando, FL.
[31] Yamamoto, H., Tagaya, M., Fukui, T. and Kawakita, M. (1988) J. Biochem. (Tokyo) 103, 452–457.