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An Attempt to Convert Noncatalytic Nucleotide Binding Site of F_1-ATPase to the Catalytic Site: Hydrolysis of Tethered ATP by Mutated α Subunits in the Enzyme

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The α and β subunits of F_1-ATPase are homologous in primary structure and have similar folding topologies. The position of the essential Glu residue in the catalytic sites which reside in the β subunits is occupied by a Gln residue in the noncatalytic nucleotide binding sites which reside in the α subunits. To test if an exchange of catalytic and noncatalytic binding sites is possible, we have replaced the Gln-Lys sequence in the noncatalytic binding site of the α subunit with Glu-Arg and, reciprocally, the Glu in the catalytic site of the β subunit with Gln. The resultant mutant $\alpha_3\beta_3\gamma$ complex lost steady-state ATPase activity. However, HPLC analysis of tryptic digests of the mutant $\alpha_3\beta_3\gamma$ complex which had been photolabeled with 2-N_3-[8-^{3}H]ATP revealed that ATP tethered to the noncatalytic binding site was hydrolyzed, indicating that a primitive catalytic ability was generated at the α subunit by the introduced Glu.

F_1-ATPase is a water-soluble, catalytic portion of H^+-ATP synthase which catalyzes ATP synthesis coupled with proton flow across membranes (reviewed in Refs. 1–4). It has a subunit structure of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ [5]. The α and β subunits have similar amino acid sequences [6] and their three dimensional structures are also similar [7]. F_1-ATPase contains six nucleotide binding sites and three sites are located mainly on the β subunits and have been defined as catalytic sites. The other three sites, whose functions have not been specified clearly and hence called noncatalytic binding sites, are located mainly on the α subunits. Using F_1-ATPase from thermophilic *Bacillus* PS3 (TF_1) which has some experimental advantage [8, 9], we have identified β-Glu190 as an essential residue from experiments of specific modification with dicyclohexyl[^{14}C]carbodiimide [10] and site-directed mutagenesis [11, 12]. This glutamic acid residue is conserved in F_1-ATPases from other sources, such as *Escherichia coli* (EF_1) and bovine heart mitochondria (MF_1) [5, 13–15], and site-directed mutagenesis of EF_1 also suggests the essential role of β-Glu 190 in catalysis (numbering of the residue is according to the sequence of TF_1 in this article) [16]. X-ray crystal analysis of MF_1 revealed that the carboxylate of this residue is hydrogen-bonded to a water molecule which is located by the side of the γ-phosphate of an ATP analogue, AMP-PNP (5′-adenylyl-imidodiphosphate), suggesting that it may act as a general base in ATP hydrolysis [17]. In the α subunit, the residue corresponding to β-Glu190 is a glutamine residue (α-Gln200) which resides in the noncatalytic binding sites (Fig. 1) and, in the three dimensional structure, α-Gln200 occupies the equivalent position to that of β-Glu190. Then, one may speculate that the lack of catalytic ability of the noncatalytic binding site is due to the lack of a catalytic carboxyl group that

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Abbreviations: F_1-ATPase, catalytic portion of H^+-translocating ATP synthase; TF_1, EF_1, and MF_1, F_1-ATPase from thermophilic *Bacillus* PS3, *Escherichia coli,* and mitochondria, respectively; HPLC, high performance liquid chromatography.

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	200
wild-type α	NMICIYVAIG**Q**K**K**ESTVATVVET
αQ200E/K201R	NMICIYVAIG**E**R**R**ESTVATVVET
	190
wild-type β	GGISVFAGVG**E**RTREGNDLYHE
βE190Q	GGISVFAGVG**Q**RTREGNDLYHE

FIG. 1. The partial amino acid sequences of the α and β subunits of F_1-ATPases from thermophilic *Bacillus* PS3 (TF_1). The residues replaced are indicated by the bold letters.

acts as a general base in ATP hydrolysis. Here, we have tested whether the replacement of the α-Gln200 by Glu converts the noncatalytic binding site to the "catalytic" site.

MATERIALS AND METHODS

The construction of the mutant β-E190Q was reported previously [11] and the α-Q200E/K201R was generated by the method of Kunkel et al. [18] using synthetic oligonucleotide, 5′-GACCGTCGACTCTCGTTCCCCGATGGC-3′. The wild-type $\alpha_3\beta_3\gamma$ and mutant $(\alpha Q200E/K201R)_3(\beta E190Q)_3\gamma$ complexes of TF_1 were expressed in the *E. coli* strain JM103△(*uncB-uncD*) and purified by the method reported previously [19]. Genetic procedures were performed as described in the manual [20].

Protein concentrations were determined by measurement of absorbance at 280 nm. 0.45 of absorbance at 280 nm was defined as 1 mg/mL of protein concentration. ATPase activity was measured at 25°C in the presence of an ATP regenerating system. An assay mixture contained 50 mM Tris-Cl (pH 8.0), 4 mM $MgCl_2$, 200 mM KCl, 2 mM ATP, 2.5 mM phospho*enol*pyruvate, 50 μg/mL pyruvate kinase (rabbit muscle), 50 μg/mL lactate dehydrogenase (pig muscle), and 0.2 mM NADH. The reaction was initiated by addition of 20 μL of the enzyme solution which contained 10 pmol of the wild-type or 200 pmol of the mutant enzyme to 1.98 mL of the assay mixture. The rate of ATP hydrolysis was monitored as the oxidation rate of NADH by measuring the absorbance decrease at 340 nm.

2-N_3-[8-^{3}H]ATP was synthesized and purified as described previously [21]. Irradiation, trypsin digestion and separation of tryptic peptides by reversed-phase HPLC were performed as follows. The lyophilized preparations of the wild-type or mutant complexes were dissolved into 250 μL of the solution (1.6 mg protein/mL) containing 145 μM 2-N_3-[8-^{3}H]ATP (2700 cpm/nmol), 2 mM $MgCl_2$, 100 μM EDTA, and 50 mM Tris-Cl (pH 7.5). The solutions were placed in wells of a 24-well tissue culture plate and irradiated with a Minerallight (Ultra Violet Products, San Gabriel, CA) which was placed 17 mm above the surface of the samples. Irradiation was carried out for 20 min at 25°C using the short-wavelength setting. Roughly, one third of total noncatalytic sites and catalytic sites in the wild-type and mutant complexes were labeled by 2-N_3-[8-^{3}H]ATP. After further incubation for 60 min at 37°C in the dark, proteins were precipitated by addition of 500 μL of saturated ammonium sulfate. The precipitates were dissolved and the ammonium sulfate precipitation was repeated again. The precipitated proteins were denatured by dissolving in 300 μL of 8 M urea, and 600 μL of saturated ammonium sulfate were added. The precipitates were dissolved in 150 μL of 8 M urea and then added were 450 μL of 50 mM Tris-Cl (pH 7.5), 100 μM EDTA, L-1-Tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin (1/40 w/w/). The solutions were incubated at 37°C for 12 hrs at which time equal amount of L-1-Tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin was added. Digestions were continued for additional 8 hrs with inserting freeze-thaw procedures twice. An aliquot of the solution was injected to a C_4 reversed-phase HPLC column (Brownlee, 220 × 4.6 mm) and developed with a gradient of CH_3CN in 0.1% HCl as follows: 0–10 min, 0%; 10–100 min, 0–24%; 100–115 min, 24–48%; 115–120 min, 48–80%. Fractions were collected at each 1 min and radioactivity of each tube was measured.

RESULTS AND DISCUSSION

As reported previously [11], $\alpha_3(\beta E190Q)_3\gamma$ complex exhibits no steady-state ATPase activity (Table 1). It cannot mediate even a single turnover of catalysis [12]. Intending conversion of the α subunit of this inactive complex into the catalytic subunit, we replaced α-Gln200 of this complex

TABLE 1
ATPase Activity of the Wild-Type and Mutant $\alpha_3\beta_3\gamma$ Complexes

Sample	ATPase activity at 25°C (*units/mg protein*)
Wild-type $\alpha_3\beta_3\gamma$	19
$\alpha_3(\beta E190Q)_3\gamma$	0.00
$(\alpha Q200E/K201R)_3(\beta E190Q)_3\gamma$	0.00

by Glu to provide a carboxylate at the noncatalytic binding site on the α subunit. In addition, α-Lys201 was replaced by Arg, because it has been suggested from mutational works of EF_1 [16] and crystal structure of MF_1 [17] that the conserved Arg residue next to the catalytic β-Glu190 also contributes to form the catalytic site. The resultant $(\alpha Q200E/K201R)_3(\beta E190Q)_3\gamma$ complex, however, showed no detectable steady-state ATPase activity, less than 0.1% of that of the wild-type complex if any (Table 1).

Then, ATP was covalently immobilized to the catalytic and noncatalytic binding sites by irradiating the complex with 2-N_3-[8-^{3}H]ATP and the hydrolysis of thus tethered ATP was examined. Previous studies have shown that, as observed for other F_1-ATPases, when TF_1 is inactivated by radioactive 2-N_3-ATP under UV irradiation, radioactive nucleotides covalently bind to β-Tyr341 and β-Tyr364 which represent nucleotide binding to catalytic sites and noncatalytic binding sites, respectively [21]. Even though the photolabeled enzyme is unable to mediate steady-state ATP hydrolysis, it can mediate hydrolysis of adenine nucleotide tethered to the catalytic sites [22]. Hydrolysis of tethered ATP to tethered ADP and even to tethered AMP was reported [22, 23]. The hydrolysis of tethered nucleotide is easily detected by the appearance of heterogeneous tryptic peptides resolved by reversed-phase HPLC each of which represents a peptide tethered by ATP, ADP or AMP. Thus, the observation of hydrolysis of tethered nucleotide by the appearance of heterogeneous peptide peaks seemed to be a sensitive, convenient method to detect the site-specific adenine nucleotide hydrolysis of the mutant $\alpha_3\beta_3\gamma$ complex.

At first, hydrolysis of tethered ATP by the wild-type $\alpha_3\beta_3\gamma$ complex was examined. Fig. 2A shows a profile of radioactive peptides resolved by HPLC from the tryptic digests of the wild-type $\alpha_3\beta_3\gamma$ complex which was photolabeled with 2-N_3-[8-^{3}H]ATP · Mg. The elution profile of the radioactivity was very similar to that of TF_1 treated with the same procedures whose radioactive peptide peaks had been assigned previously [21]. The peak at 78 min represents the peptide containing β-Tyr364 derived from the noncatalytic binding site tethered with ATP. It was eluted as a single peak indicating that ATP tethered to noncatalytic binding sites was not hydrolyzed.

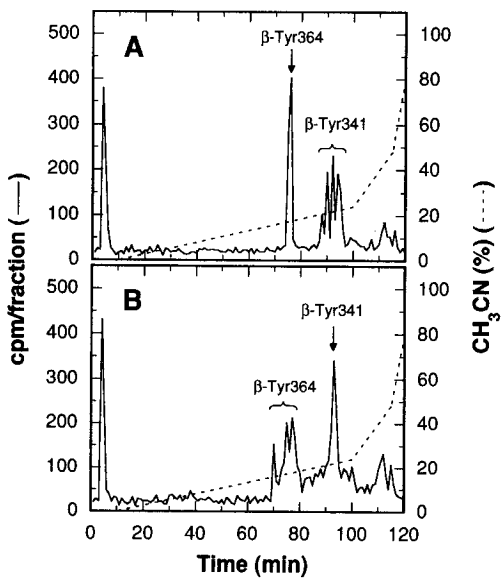

FIG. 2. Resolution of radioactive peptides in tryptic digests of the wild-type and mutant $\alpha_3\beta_3\gamma$ complex photolabeled with 2-N_3-[8-^{3}H]ATP by reversed-phase HPLC. The solution containing (A) the wild-type $\alpha_3\beta_3\gamma$ complex or (B) the $(\alpha Q200E/K201R)_3(\beta E190Q)_3\gamma$ complex was photolabeled with 2-N_3-[8-^{3}H]ATP for 20 min, incubated for 60 min in the dark, digested by trypsin, and analyzed by C_4 reversed-phase HPLC. Other conditions are described under Materials and Methods.

Several peaks at 85–94 min represent the peptides containing β-Tyr341 derived from the catalytic sites. As mentioned above, heterogeneity of the peptides should be arisen from heterogeneity of tethered adenine nucleotides and, therefore, it was concluded that ATP tethered to catalytic sites of the wild-type $\alpha_3\beta_3\gamma$ complex was hydrolyzed.

Then, we analyzed mutant $(\alpha Q200E/K201R)_3(\beta E190Q)_3\gamma$ complex which had been photolabeled with 2-N_3-[8-^{3}H]ATP · Mg by the same procedures as the wild-type complex. Fig. 2B shows a HPLC elution profile of the tryptic digests of the mutant complex. Just as opposite to that of the wild-type complex, the peptides derived from noncatalytic binding sites were eluted as several peaks while the peptide derived from catalytic sites was eluted as a single peak. This indicates that ATP tethered to the noncatalytic binding sites underwent hydrolysis to ADP and AMP whereas ATP tethered to the catalytic sites was not hydrolyzed. Thus, the carboxyl group, introduced into the α subunit at the position corresponding to Glu190 of the β subunit, is responsible for generation of a primitive catalytic ability at the noncatalytic binding site. This, in turn, provides another support for the critical role of β-Glu190 in the wild-type enzyme, that is, a general base in ATP hydrolysis. It should be mentioned, however, that this carboxyl group is not sufficient to sustain steady-state ATPase activity. This is not surprising because efficient catalytic turnover of the enzyme in general must be attained as the result of an elaborate cooperation of many residues in the enzyme.

REFERENCES

1. Futai, M., Noumi, T., and Maeda, M. (1989) *Annu. Rev. Biochem.* **58,** 111–136.
2. Senior, A. E. (1990) *Annu. Rev. Biophys. Biophys, Chem.* **19,** 7–41.
3. Penefsky, H. S., and Cross, R. L. (1991) *Adv. Enzymol.* **64,** 173–214.
4. Boyer, P. D. (1993) *Biochim. Biophys. Acta* **1140,** 215–250.
5. Yoshida, M., Sone, N., Hirata, H., Kagawa, Y., and Ui, N. (1979) *J. Biol. Chem.* **254,** 9525–9533.
6. 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.
7. Abrahams, J. P., Lutter, R., Todd, R. J., Van Raaij, M. J., Leslie, A., and Walker, J. E. (1993) *EMBO J.* **12,** 1775–1780.
8. Kagawa, Y., Sone, N., Yoshid, M., Hirata, H., and Okamoto, H. (1976) *J. Biochem.* **80,** 141–151.
9. Ohta, S., Yohda, M., Ishizuka, M., Hirata, H., Hamamoto, T., Otawara-Hamamoto, Y., Matsuda, K., and Kagawa, Y. (1988) *Biochim. Biophys. Acta* **933,** 141–155.
10. Yoshida, M., Poser, J. W., Allison, W. S., and Esch, F. S. (1981) *J. Biol. Chem.* **256,** 148–153.
11. Ohtsubo, M., Yoshida, M., Ohta, S., Kagawa, Y., Yohda, M., and Date, T. (1987) *Biochem. Biophys. Res. Commun.* **146,** 705–710.
12. Amano, T., Tozawa, K., Yoshida, M., and Murakami, H. (1994) *FEBS Lett.* **348,** 93–98.
13. Kanazawa, H., Kayano, T., Mabuchi, K., and Futai, M. (1981) *Biochem. Biophys. Res. Commun.* **103,** 604–612.
14. Kanazawa, H., Kayano, T., Kiyasu, T., and Futai, M. (1982) *Biochem. Biophys. Res. Commun.* **105,** 1257–1264.
15. Walker, J. E., Gay, N. J., Saraste, M., and Eberle, A. N. (1984) *Biochem. J.* **224,** 799–815.
16. Park, M. Y., Omote, H., Maeda, M., and Futai, M. (1994) *J. Biochem.* **116,** 1139–1145.
17. Abrahams, J. P., Leslie, A., Lutter, R., and Walker, J. E. (1994) *Nature* **370,** 621–628.
18. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) *Methods Enzymol.* **204,** 125–139.
19. Matsui, T., and Yoshida, M. (1995) *Biochim. Biophys. Acta* **1231,** 139–146.
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Jault, J. M., Kaibara, C., Yoshida, M., Garrod, S., and Allison, W. S. (1994) *Arch. Biochem. Biophys.* **310,** 282–288.
22. Jault, J. M., and Allison, W. S. (1994) *FEBS Lett.* **347,** 13–16.
23. Melese, T., Xue, Z., Stempel, K. E., and Boyer, P. D. (1988) *J. Biol. Chem.* **263,** 5833–5840.