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AROMATIC RINGS OF TYROSINE RESIDUES AT ADENINE NUCLEOTIDE BINDING SITES OF THE β SUBUNITS OF F_1-ATPase ARE NOT NECESSARY FOR ATPase ACTIVITY

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SUMMARY: Using site-directed mutagenesis, Tyr-307, Tyr-341, or Tyr-364, supposedly located at the adenine nucleotide binding site(s) of the β subunits of F_1-ATPase from the thermophilic bacterium PS3, was replaced with Phe or Cys. The $\alpha_3\beta_3$ complexes reconstituted from the α subunits and individual mutant β subunits hydrolyzed ATP. Thus, neither the hydroxyl groups nor the aromatic rings in these positions are required for ATPase activity of F_1-ATPase.

The H^+-ATP synthase of oxidative- and photo- phosphorylation is comprised of a catalytic part, F_1-ATPase, and a transmembrane proton channel, F_o (1~3). The subunit structure of F_1-ATPase is $\alpha_3\beta_3\gamma\delta\varepsilon$ and the catalytic sites reside on each β subunit or at the interfaces of the α and β subunits. The $\alpha_3\beta_3$ complex is the simplest subunit complex that has ATPase activity (4,5).

From studies using chemical modification and photoaffinity labeling it has been proposed that three Tyr residues on the β subunits, the residues conserved in the primary sequences of the β subunits of all known F_1-ATPases, are located in the adenine nucleotide binding site(s). They are β-Tyr-307, β-Tyr-341 and β-Tyr-364 according to the residue number of the β subunit of F_1-ATPase from the thermophilic bacterium PS3 (TF_1). When TF_1 was inactivated by 7-chloro-4-nitrobenzofurazan, β-Tyr-307 was specifically modified (6). The corresponding Tyr residues of the β subunits of bovine heart mitochondrial F_1-ATPase (MF_1) and

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Abbreviations : TF_1, F_1-ATPase from the thermophilic bacterium PS3; MF_1, F_1-ATPase from bovine heart mitochondria; CF_1, F_1-ATPase from spinach chloroplasts.

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spinach chloroplast F_1-ATPase (CF_1) were also modified by this reagent (7~9) or by 8-N_3-ATP(10). When MF_1 and CF_1 were inactivated by 5'-*p*-fluorosulfonylbenzoylinosine and 3'-*O*-(4-benzoyl)benzoyl-ATP, respectively, Tyr residues corresponding to β-Tyr-341 of TF_1 were modified (11,12). β-Tyr-364 of TF_1 and the corresponding Tyr residue of MF_1 were modified by 5'-*p*-fluorosulfonylbenzoyladenosine when these ATPases were inactivated by this reagent (13,14). Photoaffinity analogues, 2-N_3-ADP and 2-N_3-ATP, labeled either one or both, dependent on the labeling conditions, of two β-Tyr residues of MF_1, CF_1 and *Escherichia coli* F_1-ATPases (EF_1) corresponding to β-Tyr-341 and β-Tyr-364 of TF_1 (15~18).

The possibility that the hydroxyl groups of β-Tyr-307 and β-Tyr-364 are involved directly in the catalysis was eliminated, since Parsonage *et al.* demonstrated that EF_1 containing mutant β subunits in which one of these Tyr residues was replaced with Phe were ATPase-active (19). The complete base sequence of the TF_1 operon was determined by Kagawa and his colleagues, who also established an overexpression system for each subunit of TF_1 in *E.coli* cells (20,21). By virtue of their work and the facile reconstitution of TF_1 from the isolated subunits, TF_1 or its reconstituted subunit complexes containing genetically replaced amino acid residues at desired positions became obtainable (22,23). Here we report on the ATPase activity of the $\alpha_3\beta_3$ complex containing the mutant β subunit with replacement of one of the three Tyr residues by Phe or Cys.

MATERIALS AND METHODS

Bacterial Strains----*E.coli* strains CJ236 (*dut,ung,thi,relA;pCJ105(Cm^r)*) and MV1190 (*Δ(lac-proAB), thi, supE,Δ(sr-1recA)306::Tn10(tet^r)[F':traD36, proAB, lac, IqZΔM15]*) were used for directed mutagenesis (24). For expression of the wild type or the mutant subunits of TF_1, the strain DK8 (*balR, thi, rel I, HfrPOI, ΔuncB~C, ilv::Tn10*) which lacks the genes of F_1, was used.

Directed Mutagenesis----The TF_1-β gene cloned in the plasmid pUC118 had its own Shine-Dalgarno sequence down stream of *lac* promotor and the transformed *E.coli* cells by this plasmid overproduced the intact β subunit of TF_1. The synthetic oligonucleotides used for mutagenesis are listed in Table I. In addition to the base sequence corresponding to an amino acid residue to be changed, oligonucleotides were designed to contain sequences to create a new restriction site which was easily detectable with agarose gel electrophoresis. An uracil-containing single-stranded template for mutagenesis was prepared by infecting uracil-incorporating mutant CJ236 cells as described by Kunkel *et al.* (25). Single- stranded DNA of pUC118 containing the TF_1-β gene was mixed with a phosphorylated oligonucleotide with a one to twenty molar ratio in an annealing buffer (200 mM Tris HCl, pH 7.4, 20 mM $MgCl_2$, 500 mM NaCl) at a final volume of 10μl, and the mixture was heated in boiling water for 1 min. The reaction mixture was replaced in a water bath at 70°C and was allowed to cool to 30°C at a rate of approximately 1°C per min. Then, the reaction mixture was placed in an ice-water bath and 1μl of

Table I. Sequences of Mutagenic Oligonucleotides

Mutation	Mutagenic Oligonucleotide	Change of Restriction Sites
β(wild)	5′--- --- GTC GGC CGG GAC GTA AAT CGG TT- ---3′	Rsa I
β(Y307F)	--- --- --- T-- -A- --- --- --	1108 → 781 + 327
β(Y307C)	--- --- --- T-- -C- --- --- --	
β(wild)	5′-AG CGG GTC AAC GGC CGG ATA AAT CCC CAT C--3′	Pst I
β(Y341F)	-- --- --- --- T-- A-- -A- --- --- --- -	4713 → 4284 + 429
β(Y341C)	-- --- --- --- T-- A-- -C- --- --- --- -	
β(wild)	5′--- CAC TTT GCG GGC GAC TTG ATA ATG CTC CT-3′	BssH II
β(Y364F)	--- --- --- C-- --- --- -A- --- --- --	4713 → 3720 + 993
β(Y364C)	--- --- --- C-- --- --- -C- --- --- --	

The unchanged bases are shown as dashed lines(-).

a synthesis buffer (4 mM dNTP, 7.7 mM ATP, 175 mM Tris sulfate, pH 7.4, 37.5 mM $MgCl_2$, 15 mM DTT), 3 units of T4 DNA ligase, and 1 unit of *E.coli* DNA polymerase I were added. The reaction mixture was incubated on ice for 5 min, then at 25°C for 5 min, and finally at 37°C for 90 min, and was used for transformation of the *E.coli* strain MV1190. This strain has an active uracil N-glucosylase which will inactivate the uracil-containing parental strand, thus enriching the mutant plasmids. Mutant plasmids were selected by changed restriction patterns predicted for mutants (Table I) and confirmed by DNA sequencing.

Purification of TF_1 and Its Subunits----The growth, harvesting, and lysis of the thermophilic bacterium PS3, and the purification of TF_1 were carried out as described in (26). The wild type α and β subunits of TF_1 were expressed in the *E.coli* DK8 strain individually and each subunit was isolated as described in previous reports (22,23). The purified proteins were stored in 75% saturated ammonium sulfate suspension at 4°C. The mutant β subuints were expressed and isolated with the same procedures.

Reconstitution----Reconstitution of the subunit complex, $\alpha_3\beta_3$, from each subunit and purification of the complexes with a HPLC gel filtration column (G3000SWXL, Tosoh Co. Tokyo) was carried out as described in (4). The complexes were stored in 75 % ammonium sulfate suspension at 4°C.

Assays of ATPase Activity----ATP hydrolysis catalyzed by the enzyme was monitored at 25°C by a coupled enzyme assay. Reaction mixtures contained 50 mM Tris sulfate, pH8.0, 2mM $MgCl_2$, 200mM Na_2SO_4, 10mM KCl, 6.8% ammonium sulfate, 5mM phospho*enol*pyruvate, 0.2mM NADH, 10μg/ml pyruvate kinase, 10μg/ml lactate dehydrogenase, and indicated concentrations of ATP. Reactions were initiated by the addition of the enzyme. When most of the NADH in a reaction mixture was oxidized, new NADH was added to continue the reaction. Protein concentrations were measured by using Coomssie brilliant blue dye reagent (27).

RESULTS

All of the $\beta_{(mutant)}$ subunits retained the ability to reconstitute $\alpha_3\beta_3$ complexes with the α subunit. The complexes were purified and their ATPase activities were measured. Fig.1 shows the traces of NADH oxidation coupled with ATP hydrolysis catalyzed by TF_1 and the complexes. It is obvious that all of the $\alpha_3\beta_{(mutant)3}$

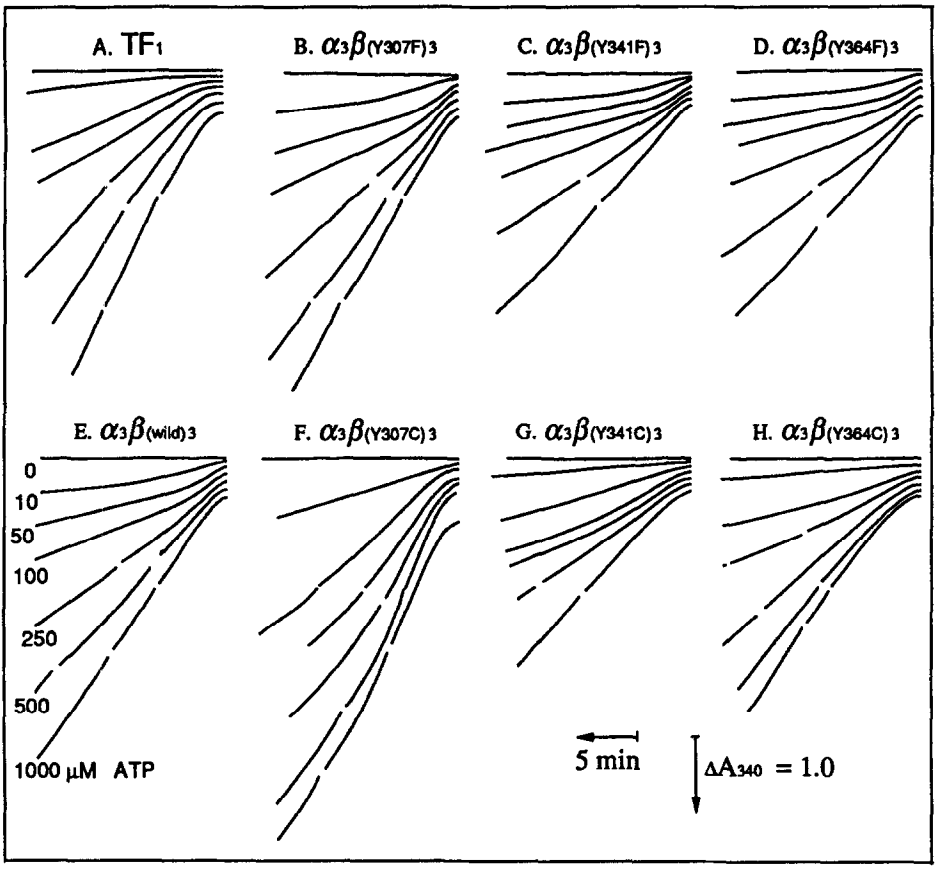

Fig.1. Time course of ATP hydrolysis by TF_1 and the $\alpha_3\beta_{(wild)_3}$ and $\alpha_3\beta_{(mutant)_3}$ complexes. Oxidation of NADH coupled with ATP hydrolysis was monitored photometrically at 340nm. The trace was started at 20s after the addition of the enzyme. The amount of enzyme in a 660μl of reaction mixture was; TF_1, 5.5μg; $\alpha_3\beta_{(wild)_3}$, 63μg; $\alpha_3\beta_{(Y307F)_3}$, 64$\mu$g; $\alpha_3\beta_{(Y341F)_3}$, 61$\mu$g; $\alpha_3\beta_{(Y364F)_3}$, 63$\mu$g; $\alpha_3\beta_{(Y307C)_3}$, 37$\mu$g; $\alpha_3\beta_{(Y341C)_3}$, 69$\mu$g; $\alpha_3\beta_{(Y364C)_3}$, 60$\mu$g. When most NADH in the reaction mixture was oxidized and the absorbance at 340nm became lower than 0.4, 7μl of 20mM NADH was added. The periods for this procedure are shown as breaks in the traces. Other conditions of the assays were the same as described in MATERIALS AND METHODS except that the reaction mixture for TF_1 did not contain ammonium sulfate. Concentrations of ATP are 0, 10, 50,100, 250, 500 and 1000μM from top to bottom. Scales of time and absorbance are indicated in the Figure.

complexes are ATPase-active. The initial rates of ATP hydrolysis were non-linear. ATP hydrolysis by TF_1 exhibited a lag period which became shorter with increasing ATP concentrations (Fig.1 A) (28). The reaction kinetics of ATP hydrolysis by the complexes containing the $\beta_{(Y364C)}$ subunits or one of the three $\beta_{(Y\rightarrow F)}$ mutant subunits are similar to that of the $\alpha_3\beta_{(wild)_3}$ complex. At low ATP concentrations, a faster, linear initial rate preceded a slower, constant rate of hydrolysis. At high ATP concentrations, an initial lag period appeared (Fig.1 B~E, H). The $\alpha_3\beta_{(Y341C)_3}$ complex showed a somewhat more pronounced lag period (Fig.1 G). The reaction

Table II. ATPase Activities of the $\alpha_3\beta_{(mutant)3}$ Complexes

Complex	Activity	
	unit/mg	%
$\alpha_3\beta_{(wild)3}$	0.41	100
$\alpha_3\beta_{(Y307F)3}$	0.46	112
$\alpha_3\beta_{(Y307C)3}$	1.3	317
$\alpha_3\beta_{(Y341F)3}$	0.35	85
$\alpha_3\beta_{(Y341C)3}$	0.26	63
$\alpha_3\beta_{(Y364F)3}$	0.33	80
$\alpha_3\beta_{(Y364C)3}$	0.39	95

One unit of activity is defined as the activity which hydrolyzes 1μ mol of ATP per min. The rates of ATP hydrolysis at 6 min after starting the reaction are shown as the values of activities. Reaction mixtures contained 1mM ATP. Under the same conditions except omitting ammonium sulfate, the activity of TF_1 was 5.3 unit/mg.

kinetics were even more complicated for the $\alpha_3\beta_{(Y307C)3}$ complex. In this case, a lag period preceded accelerated hydrolysis which was followed by slower hydrolysis (Fig.1 F). The factors underlying the non-linear reaction kinetics of the complexes are unknown. Since once the complexes attained a constant rate of hydrolysis, which lasted for several minutes, the possibility that the complexes dissociated into subunits during assay is not likely. However, such an argument is not valid for the $\alpha_3\beta_{(Y307C)3}$ complex which did not attain a constant rate of hydrolysis throughout the period of measurement. Since the complexes exhibited complicated kinetics, rather than estimating Km and Vmax values, the rates of ATP hydrolysis obtained from the slope of NADH oxidation at 6min after initiation of the reaction at an ATP concentration of 1mM are compared (Table II). All of the $\alpha_3\beta_{(mutant)3}$ complexes have more than 50% of the activity of the $\alpha_3\beta_{(wild)3}$ complex. The $\alpha_3\beta_{(Y307F)3}$ and $\alpha_3\beta_{(Y364C)3}$ complexes have comparable activities with the $\alpha_3\beta_{(wild)3}$ complex, and the $\alpha_3\beta_{(Y307C)3}$ complex has even higher activity than that of the $\alpha_3\beta_{(wild)3}$ complex.

DISCUSSION

Parsonage *et al.* showed that the hydroxyl groups of two Tyr residues, corresponding to β-Tyr-307 and β-Tyr-364 of TF_1, are not essential for ATPase activity of EF_1 (19). The observations reported here confirm their conclusion and extend it to β-Tyr-341, which it has been proposed is located at a catalytic site

(11,18). The enzymatic characteristics, dependence of the activity on pH, temperature, metal cations, etc., of the complexes containing these $\beta_{(Y\rightarrow F)}$ mutant subunits are similar to those of the complex containing $\beta_{(wild)}$ subunit (M.Yoshida, unpublished results). Furthermore, this work also revealed that even the aromatic rings of these Tyr residues are not essential for the ATPase activity. However, it should be noted that reaction kinetics, as shown in Fig.1, are changed by these mutation, especially for $\beta_{(Y307C)}$. The apparent contradiction between the results reported here and those obtained from chemical and photo-affinity labeling can be reconciled by the interpretation that these Tyr residues are located at the adenine nucleotide binding cleft(s) but are not involved in catalysis itself. Spatial occupation of the cleft(s) by a bulky labeling group may cause interference of catalysis. The fact that these Tyr residues, though conserved in the β subunits of all known F_1-ATPases, are not conserved in homologous catalytic subunits of H^+-ATPases of archaebacteria and eukaryotic endomembrane vacuoles (29), favors of this interpretation.

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