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Stable Structure of Thermophilic Proton ATPase Beta Subunit[1,2]

Yasuo KAGAWA,*,[3] Morio ISHIZUKA,** Toshio SAISHU,*** and Shinichi NAKAO*

*Departments of Biochemistry and ***Anesthesiology, Jichi Medical School, Minamikawachi, Tochigi 329-04, and **Department of Industrial Chemistry, Faculty of Science and Engineering, Chuo University, Bunkyo-ku, Tokyo 112

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F_1-ATPase is the major enzyme for ATP synthesis in mitochondria, chloroplasts, and bacterial plasma membranes. F_1-ATPase obtained from thermophilic bacterium PS3 (TF_1) is the only ATPase which can be reconstituted from its primary structure. Its β subunit constitutes the catalytic site, and is capable of forming hybrid F_1's with *E. coli* α and γ subunits. Since the stability of TF_1 resides in its primary structure, we cloned a gene coding for TF_1, and the primary structure of the β subunit was deduced from the nucleotide sequence of the gene to compare the sequence with those of β's of three major categories of F_1's; prokaryotic membranes, chloroplasts, and mitochondria. The following results were obtained. 1) Homology: The primary structure of the $TF_1\beta$ subunit (473 residues, $M_r = 51{,}995.6$) showed 89.3% homology with 270 residues which are identical in the β subunits from human mitochondria, spinach chloroplasts, and *E. coli*. It contained regions homologous to several nucleotide-binding proteins. 2) Secondary structure: The deduced α-helical (30.1%) and β-sheet (22.3%) contents were consistent with those determined from the circular dichroism spectra. Residues forming reverse turns (Gly and Pro) were highly conserved among the $F_1\beta$ subunits. 3) Substituted residues and stability of TF_1: We compared the amino acid sequence of the $TF_1\beta$ subunit with those of the other $F_1\beta$ subunits mentioned above. The observed substitutions in the thermophilic subunit increased its propensities to form secondary structures, and its external polarity to form tertiary structure. 4) Codon usage: The codon

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[3] To whom correspondence should be addressed.

Abbreviations: FoF_1, proton-translocating ATPase; $TFoF_1$, thermophilic FoF_1; Fo, proton channel portion of proton translocating ATPase; TFo, thermophilic Fo; F_1, catalytic portion of proton-translocating ATPase; CF_1, chloroplast F_1; EF_1, *E. coli* F_1; HF_1, human mitochondrial F_1; TF_1, thermophilic F_1; F_1, the β subunit of F_1; bp, base pairs; SSC, NaCl-sodium citrate buffer.

usage of the $TF_1\beta$ gene was found to be unique. The changes in codons that achieved these amino acid substitutions were much larger than those caused by minimal mutations, and the third letters of the optimal codons were either guanine or cytosine, except in codons for Gln, Lys, and Glu.

Proton-translocating ATPase (FoF_1) plays a central role in oxidative and photosynthetic phosphorylation (*1*). FoF_1 consists of a catalytic portion, called F_1 (*2*) and a proton channel portion, called Fo (*3*). An operon coding for FoF_1 of *Escherichia coli* (*4*, *5*) and genes for some subunits of chloroplasts (*6–8*), yeast mitochondria (a corrected portion of the sequence of the β subunit was communicated by Dr. M. Takeda) (*9*) and *Rhodopseudomonas blastica* (*10*) have been sequenced. F_1 obtained from thermophilic bacterium PS3, called TF_1 (*11*, *12*), has the following important unique properties. 1) It can be reconstituted from its five subunits after their higher-order structures have been completely destroyed with urea and guanidium chloride (*12*, *13*). 2) It is resistant to many drastic conditions and a wide range of ionic gradients (*14*). 3) It does not contain bound nucleotides (*15*) or tightly bound Mg. A study of the cation-dependent diastereoisomer preference of TF_1 revealed that its true substrate is a Δ, β, γ ATP-Mg complex (*16*). The specific interactions with 8-azido ATP (*17*) and 3′-arylazido-8-azido ATP (*18*) may also be useful in analyzing its catalytic center. 4) It can be crystallized with or without nucleotides (*19*, *20*). 5) Conformational studies of both TF_1 and its subunits using IR spectroscopy (*21*) and NMR spectroscopy at high temperature (*12*) are possible.

There are many hypotheses on the thermostability of proteins (*22*, *23*) but homologous proteins obtained from mitochondria, chloroplasts, and mesophilic bacterial thermophilic plasma membranes have not yet been compared at both peptide and nucleotide levels. The β subunit of TF_1 is supposed to constitute a catalytic center judging from the results of reconstitution studies (*12*, *13*) and partial peptide sequencing of the dicyclohexylcarbodiimide-binding site of TF_1 (*24*). For testing these hypotheses on thermostability, the amino acid sequence of the thermophilic β subunit must be aligned correctly with the corresponding sequences of other β subunits to locate substituted residues. Fortunately, very high homology in the amino acid sequences of the β subunits obtained from several sources has been reported (*10*, *25*, *26*). Moreover, $TF_1\beta$ is the special thermophilic subunit which can form active hybrid F_1's with *E. coli* subunits (*27*), and thus it must be very similar in tertiary structure to mesophilic $F_1\beta$ subunits. Recently, the $F_1\beta$ subunit of human mitochondria has been sequenced in this laboratory (*28*), and thus $F_1\beta$ of all three categories of biomembranes can be compared with TF_1. In this paper, we report cloning of the genes for thermophilic FoF_1, the sequence of $TF_1\beta$ subunit and the analysis of its stable structure.

MATERIALS AND METHODS

Strains and Media—Thermophilic bacterium PS3 (*13*) was grown on YPS medium (0.4% yeast extract, 0.8% bactopeptone, and 0.3% NaCl). *Escherichia coli* strain JM 101 (*Δ lac-pro, thi, sup* E/F′ *tra* D36, *pro* AB, *lacI*^{q}Z *Δ* M15) used for cloning experiments in M13 phage was selected by growth on plates of glucose/minimal medium and grown on 2×TY medium (1.6% bactotryptone, 1% yeast extract, 0.5% NaCl). *E. coli* strain DH1 [*F*$^-$, *recA1, endA1, gyrA96, thi-1, hsdR17* (r_K^-, m_K^+), *supE44, relA1?*, λ^-] was also used for cloning experiments with pBR322 (*29*). When necessary, ampicillin or tetracycline was added in LB medium to final concentrations of 40 μg/ml and 25 μg/ml, respectively.

Preparation of Probes—Hybrid plasmid pFT 1503 containing the *Pst*I-*Eco*RI fragment (790 base pairs) of the *E. coli* $F_1\beta$ gene, and pMCR 533 carrying the *Hin*dIII-*Eco*RI fragment (2,356 base pairs) of the *E. coli* Fo gene were obtained from Dr. H. Kanazawa and Dr. M. Futai, Okayama University (*4*). Hybrid plasmid pTB 17 carrying the *Bam*HI-*Cla*I fragment (595 base pairs) of the tobacco chloroplast $F_1\beta$ gene was a gift from Dr. M. Sugiura, Nagoya University (*8*). These inserted DNAs were digested with the above-mentioned

restriction enzymes, isolated by agarose gel electrophoresis, and labeled with ^{32}P by nick translation (*29*). Mixed oligonucleotides, 14 mer corresponding to NH_2-Met-Phe-Ile-Gly-Val-OH (part of the amino acid sequence of the TFo-c subunit) (*30*) and 13 mer corresponding to NH_2-Tyr-His-Glu-Met-OH (part of the TF_1-β subunit) (*24*), were synthesized with a DNA synthesizer (Applied Biosystems 380A). These synthetic DNAs were labeled with ^{32}P using T4 polynucleotide kinase (*29*).

Identification of the $TFoF_1$ Gene—PS3 genomic DNA was prepared by the method of Marmur (*31*), and Berns and Thomas (*32*), and digested with various restriction endonucleases. DNA fragments were isolated by electrophoresis, and subjected to Southern hybridization (*33*) using the probes described above and 6×SSC solution (*29*) at 60°C. The resulting double-stranded DNA on a nitrocellulose filter was washed with 0.2×SSC solution at 37 to 40°C. Hybridization was not successful at higher temperatures.

Cloning of Genes Coding for $TFoF_1$—Since the probes used were either genes of *E. coli* FoF_1 or similar DNA described in "MATERIALS AND METHODS," it was essential to use M13 phage containing the thermophilic DNA to separate contaminating *E. coli* FoF_1 gene. Thermophilic DNA (100 μg) was digested with *Sal*I and the resulting DNA fragment (200 ng, 755 bp) was collected by agarose gel electrophoresis. M13 mp 8 replicative form DNA (1 μg) was also digested with *Sal*I, then treated with calf intestinal alkaline phosphatase, and the DNA was purified by electrophoresis on low melting agarose gel. PS3 DNA (200 ng, the 755 bp *Sal*I fragment) and 20 ng of the *Sal*I digested DNA of 8 mp were ligated, transformed into *E. coli* JM 101, and plated onto an H plate (H medium: 1% bactotryptone and 0.8% NaCl). Infected cells from recombinant colorless plaques were grown on 2×TY medium (2 ml) to produce single-stranded templates. Eighty-three single-stranded recombinant DNAs were purified, subjected to dot blot hybridization (*34*) at 60°C in 6×SSC, and washed with 0.2×SSC at 40°C, using ^{32}P-labeled *E. coli* $F_1\beta$ DNA obtained by nick translation (*29*) as a probe. Only one recombinant gave a positive spot. The replicative form M13 phage prepared from the positive single-stranded recombinant DNA was digested with *Sal*I. The 755 base pair fragment (inserted PS3 DNA) was isolated and recloned into the *Sal*I site of pBR 322 for amplification. The *Pst*I fragment (9.3 kilo bp) carrying most of the $TFoF_1$ gene operon was covered by 'gene walking' using the 755 base pair fragment containing half the $TF_1\beta$ gene.

Sequencing of DNA and Peptide—DNA was sequenced by both the method of Maxam and Gilbert (*35*) as modified for thermophilic DNA (*36*), and the method of Sanger *et al.* (*37*). The amino acid composition (*38*) and partial peptide sequence of $TF_1\beta$ (*24*) were described previously, but the peptide sequences of the N-terminal and several other portions were determined using a peptide sequencer (Applied Biosystems 470A, program for 470A, 25 cycles, 5 nmol) equipped with a quantitative HPLC SP4200 (yield 90%). The sequence data were analyzed with software (Genetyx, Tokyo) adapted for a PC9801 computer (Nippon Electric Co., Tokyo).

Reagents—The restriction endonucleases *Aat*II, *Apa*I, *Ava*I, *Bst*EII, *Dra*I, *Hin*cII, *Hpa*I, *Nci*I, *Nco*I, *Sal*I, *Hin*dIII, *Pst*I, *Sma*I, and *Xho*I were purchased from Takara Shuzo (Kyoto, Japan) and *Cla*I was purchased from Japan Bio-Rad Laboratory (Tokyo). Alkaline phosphatase (*E. coli* A19), polynucleotide kinase, and other enzymes were also obtained from the above companies. Carrier-free $^{32}P_i$ was obtained from the Japan Atomic Energy Institute (Tokai, Japan), and [α-^{32}P]dCTP from Amersham (Amersham, England). Other materials and methods used were as described previously (*36*).

RESULTS AND DISCUSSION

Restriction Map of the Gene of $TFoF_1$—The sequencing strategy is shown in Fig. 1. The *Pst*I thermophilic DNA fragment of 9.3 kilo base pairs hybridized with probes of both Fo and F_1. The probes for Fo used were the 14 mer synthetic oligonucleotide corresponding to the c subunit of TFo (described in "MATERIALS AND METHODS"), and DNA carried in pMCR 533 (described in "MATERIALS AND METHODS"). The *Pst*I fragment also hybridized with the probe for F_1 derived from *E. coli*, but not with that from chloroplasts. This difference may be because the third letters of codons of chloroplast DNA have high

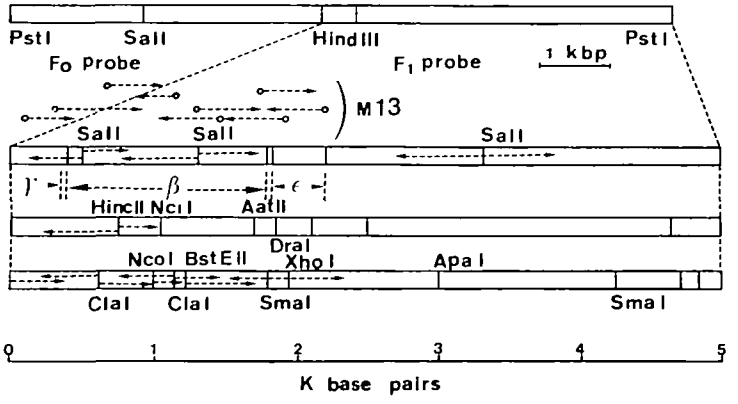

Fig. 1. Restriction map of thermophilic ATPase. Broken arrows with open circles represent positions of sequences determined with M13 phage by the method of Sanger *et al.* (*37*). Broken arrows starting from lines (restriction sites) in boxes represent positions of sequences determined by the method of Maxam and Gilbert (*35*). The fragments were 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, and in the case of *Sal*I and *Cla*I sites, the fragments were also 3′ end-labeled using Klenow polymerase and [α-^{32}P]dCTP. β, γ, and ε in the box represent the reading frames for the β, γ, and ε subunits, respectively.

contents of adenine and thymine (*6–8*), while those of thermophilic DNA are rich in guanine and cytosine (*36*). The *Sal*I fragment (755 bp, Fig. 1, second box, left *Sal*I-*Sal*I fragment) hybridized with the F_1 probe of *E. coli* but not with Fo probes.

Primary Structure of the $TF_1\beta$ Subunit—The nucleotide sequence of the gene for the $TF_1\beta$ subunit and its flanking regions are shown in Fig. 2. The Shine-Dalgarno sequence (AGGAGG) (*39*), which is complementary to a sequence near the 3′ end of 16S rRNA, is present 8–13 base pairs upstream from the translation initiation codon and may operate as a ribosome-binding site. The 5′ upstream region of the Shine-Dalgarno sequence is the C-terminal segment of the γ subunit, and the 3′ downstream region, the N-terminal segment of the ε subunit. Both flanking sequences were deduced from the homologies with subunits of other origins and partial amino acid sequences of the purified subunits.

The amino acid sequence was deduced from the nucleotide sequence and it was identical to the partial amino acid sequences determined protein-chemically in this study (#3 to 19) or reported in previous studies (#186 to 191 (*24*); #192 to 202 (*40*)). The amino acid composition deduced from the nucleotide sequence was also very similar to

TABLE I. Amino acid composition of the thermophilic $F_1\beta$ subunit. The values are residues per subunit.

Amino acid	Analysis of	
	DNA	Protein
Asp	29	[41.7
Asn	11	[
Thr	29	27.7
Ser	17	16.0
Glu	41	[66.1
Gln	21	[
Pro	23	25.5
Gly	43	44.2
Ala	39	38.3
Cys	0	0
Val	42	41.1
Met	14	12.4
Ile	35	32.4
Leu	40	41.6
Tyr	12	12.4
Phe	17	16.8
His	12	11.2
Trp	0	0
Lys	20	19.0
Arg	28	25.8
Total	473	472.2
M_r	51,995.60	

Fig. 2. DNA sequence and deduced amino acid sequence of the gene for the thermophilic $F_1\beta$ subunit and its flanking regions. The underlined peptide sequences are homologous regions; β subunit, common to those of CF_1 (*6*, *7*), HF_1 (*28*), EF_1 (*4*, *5*), and F_1 of *R. blastica* (*10*); δ subunit, to EF_1 (*4*, *5*) and F_1 of *R. blastica* (*10*); and ϵ subunit, to EF_1 (*4*, *5*), CF_1 (*6*, *7*) and F_1 of *R. blastica* (*10*).

that obtained by amino acid analysis of the subunit (Table I). The molecular weight of $TF_1\beta$ was calculated to be 51,995.6, which is close to the values determined by sedimentation equilibrium (51,000) and gel filtration (52,000) (*38*).

Homology with Other F_1s and Nucleotide-Binding Proteins—The amino acid sequence of TF_1 can readily be aligned with those of $F_1\beta$ obtained from spinach chloroplasts (CF_1) (*6*), human mitochondria (HF_1) (*28*), and *E. coli* (EF_1) (*4*, *5*) (Fig. 3). The homologies of TF_1 with the β subunits obtained from CF_1, HF_1, and EF_1 were 66.0, 67.7, and 65.3%, respectively. Since $TF_1\beta$ is exchangeable with $EF_1\beta$ to reconstitute the active hybrid F_1s, the homologous segment must be important in both function and subunit interaction. There are 270 identical residues in the β subunits in CF_1, HF_1, and EF_1, and 241 of these residues (89.3%), which are boxed in Fig. 3, were also identical in TF_1. The high homologies among these subunits were also confirmed by Harr plots. This high homology is probably a consequence of the location of the active site of F_1 on the β subunit, as discussed in detail previously, especially for $EF_1\beta$ (*4*, *5*, *26*, *27*). $TF_1\beta$ shows the following homologies to nucleotide-binding proteins (num-

bers are amino acid residues from the N-terminus): a region (#144–165) homologous to regions of oncogene ras p21 (#5–36), adenylate kinase AMP-binding pocket (#15–21), rabbit myosin S_1 head (#165–193), and *rec*A protein (#52–84); a region (#193–206) homologous to *rec*A protein (#266–282); and a region (#235–263) homologous to ATP/ADP antiporter (#283–291) and phospho-fructokinase (#93–111). A region of acid-base clusters (*41*) is also conserved in $TF_1\beta$ (#390–405). A metal-reactive site of *E. coli* $F_1\beta$ (Ser^{174}) (*42*) corresponds to Ser^{183} of $TF_1\beta$, but one of the 8-azido ATP-binding sites of beef $F_1\beta$ (*43*) (corresponding to Lys^{297} of $TF_1\beta$) was not detected in $EF_1\beta$. The dicyclohexylcarbodiimide-binding site of both $EF_1\beta$ and beef F_1 (corresponding to Glu^{190} of $TF_1\beta$) is distant from that of $TF_1\beta$ (Glu^{224}) (*40*). In short, most of the reactive sites found in other $F_1\beta$'s were also found in the deduced sequence of $TF_1\beta$ (Fig. 2).

Amino Acid Substitutions in $TF_1\beta$ for Stability —Renaturation of $TF_1\beta$ (*13*) clearly revealed that its reconstitutability and stability are due to its primary structure. Other $F_1\beta$'s become insoluble after their denaturation (Y. Kagawa, unpublished experiments). As shown in Fig. 3 and Table II, comparing the β subunits obtained from TF_1, CF_1, HF_1, and EF_1, residues forming reverse turns are well conserved (Gly=92.3%; Pro=82.0%, Tyr=75.0%), and presumably contribute to keeping the tertiary structure of TF_1 homologous with those of other $F_1\beta$'s. By comparison of thermophilic

TABLE II. Matrix of amino acid changes in aligned sequences of thermophilic $F_1\beta$ and mesophilic $F_1\beta$'s. The table shows, for example, that there are five positions in the aligned sequences where an asparagine in $CF_1\beta$, $HF_1\beta$, and/or $EF_1\beta$ changes to an alanine in $TF_1\beta$ (Fig. 3). Terms along the

Residues in $HF_1\beta+CF_1\beta+EF_1\beta$	Residues in the β subunit of							
	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly
Ala	80	4	1	1		1	6	1
Arg	1	58	2	1		1	2	1
Asn	5	2	17	6		1	1	1
Asp	4	1	1	53		2	5	1
Cys	1							
Gln	5	1	2	3		29	8	
Glu	3	1	1	9		5	77	3
Gly	3			2		1	2	121
His	1					1		
Ile	3					1		
Leu		1				1	3	
Lys	5	5		1		7	3	
Met	1					3	1	
Phe		1						
Pro		2	4	2		1	3	
Ser	6	3		2		4	8	1
Thr	2	5				1	1	
Trp				1				
Tyr		3						
Val			3	3		4	2	2
Homology (%)	66.6	66.6	58.0	63.0		46.0	63.1	92.3

and mesophilic (several species) dehydrogenases, Argos *et al.* (*22*) found that Gly, Ser, Ser, Lys, and Asp in mesophiles are generally replaced by Ala, Ala, Thr, Arg, and Glu, respectively, in thermophiles. However, in $F_1\beta$'s, these changes at corresponding residues were very few, with the same extent of amino acid substitutions in the reverse directions; *e.g.* only 2 Ser in the mesophilic β's are replaced by Thr in $TF_1\beta$, with the same substitution in the reverse direction (Table II).

The secondary structure of TF_1 was not lost until about 86°C (86% of the $[\theta]_{222}$ and 18% of ATPase activity remained after the treatment) (*11*). The reconstitutability of the secondary structure of TF_1 (*20*, *21*) must be favored by the increase in formers of both helices and sheets by the substitutions. The secondary structure of $TF_1\beta$ was deduced by Chou and Fasman's procedure (*44*). (Fig. 4). The α-helix and β-sheet contents of $TF_1\beta$ determined by circular dichroism spectroscopy were 34 and 23%, respectively (*38*), which are close to the calculated contents of 30.1 and 22.3%. The calculated values for $EF_1\beta$ were 27.2 and 13.7%, respectively (*4*). Fourier transform infrared spectroscopy of $TF_1\beta$ also revealed the presence of about 50% of the secondary structure containing a large amount of antiparallel β-sheet (a sharp peak at 1640 cm^{-1}) (*21*), which, for example, appears as a β-meander (Fig. 4, residues #204–260). The substituted residues of $TF_1\beta$ in the regions of α-helices and β-sheets were mostly formers of these structures with higher propensities (*44*) than those

diagonal show the number of times that a given amino acid is not changed. The terms in the last line are the percentage of the unchanged residues per total residues.

thermophilic F_1 ATPase											
His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
3	3	1	4	2		4	4	3			
1	2		5		3			1			
3		1	1				2	2			
			2			1	1				3
		1									
2		3	5	1							2
		3	7			1	1				2
2			4			2	2	3			
17								1			1
	64	3	2	4							13
2	3	95		11						5	4
1	1	2	28	5		1	4			1	
1	1	7	1	21	5	1					1
2		2			36						
		1			1	55				1	1
2			1			2	35	2			2
	1		2				2	67			4
	2				6					24	
	25	2		1				8		1	84
47.2	62.7	78.5	45.1	46.6	70.5	82.0	68.6	77.0		75.0	69.4

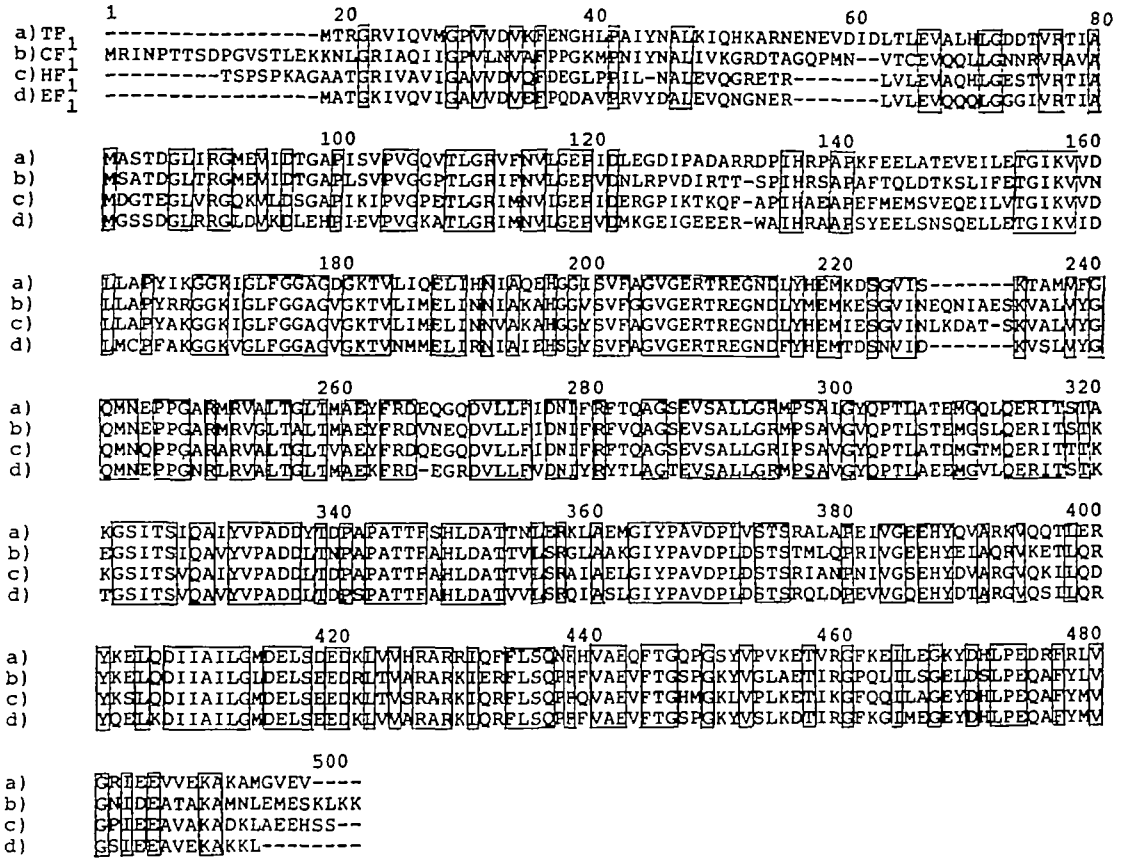

Fig. 3. Alignment of the protein sequences of the $F_1\beta$ subunits from a) thermophilic bacterium PS3 (this work), b) spinach chloroplasts (*6*), c) human mitochondria (*28*), and d) *E. coli* (*4*, *5*). Identical residues are boxed. The signal presequence of F_1 of human mitochondria (*28*) has been removed.

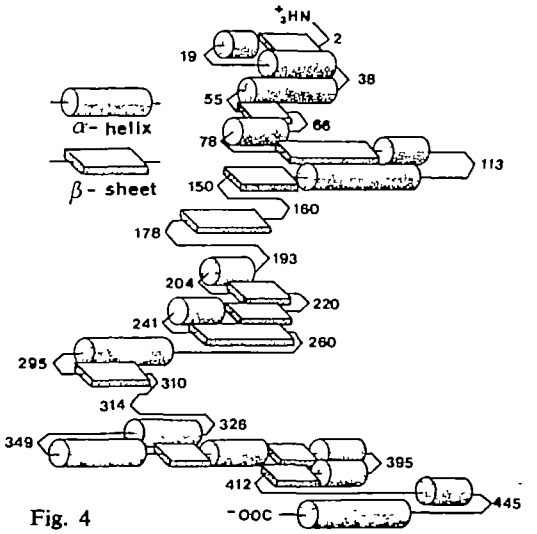

Fig. 4

of mesophilic $F_1\beta$'s (Table III).

A role of ionic bonds in thermophilic proteins has been pointed out (*23*) and $TF_1\beta$ was rendered unstable and less reconstitutable by acetylation (*45*) to reduce polar residues. In fact, the $TF_1\beta$ specific insertion (Glu^{39} to Asp^{45}) contains 4 carboxylic acid residues, and there are many substitutions of polar residues around reverse turns (Fig. 4, Table III) which may enhance the external polarity of $TF_1\beta$. As pointed out by Kuntz (*46*), turns occur on the surface of protein molecules

Fig. 4. Secondary structure of the $F_1\beta$ subunit of thermophilic bacterium PS3. The structure was deduced from the amino acid sequence by the method described by Chou and Fasman (*44*) using a GENETYX program for an NEC PC9801 computer.

lacking disulfide bonds. As shown in Fig. 5, the patterns of hydrophilicity, deduced by the method of Kyte and Doolittle (*47*), of $TF_1\beta$, $EF_1\beta$, and $HF\beta$ (without signal peptide) are very similar, except for Arg^{37}-Asp^{145}, Glu^{106}-Asp^{116}, Lys^{334}-Glu^{337}, and the C-terminal of $TF_1\beta$. On the other hand, there are some substituted residues that are more hydrophobic than in other $F_1\beta$'s, in non-turn regions. Frequent substitutions of mesophilic Val with Ile (24%, Table II) may also increase internal packing and hydrophobicity (*22*). Crystallography of TF_1 (*19*) and hydrodynamic analysis of $TF_1\beta$ (*38*) revealed that the structure of $TF_1\beta$ is globular. Folding of the secondary structure was also shown by the presence of 20% of "hard core," which was resistant to deuterium-hydrogen exchange treatment for 18 h (*21*). This hard core may be stabilized by the internal hydrophobicity formed by the hydrophobic substituted residues. No external aromatic residues were detected by an NMR study of $TF_1\beta$ at 50°C (unpublished result, *12*). The physical properties of $TF_1\beta$ could not be compared with those of other subunits because other β subunits are denatured rapidly. However, the tertiary structure of the β subunits from EF_1 must be homologous to that of $TF_1\beta$, because these subunits can form hybrid F_1's (active ATPase) (*27*), and residues forming reverse turns are conserved (Table II).

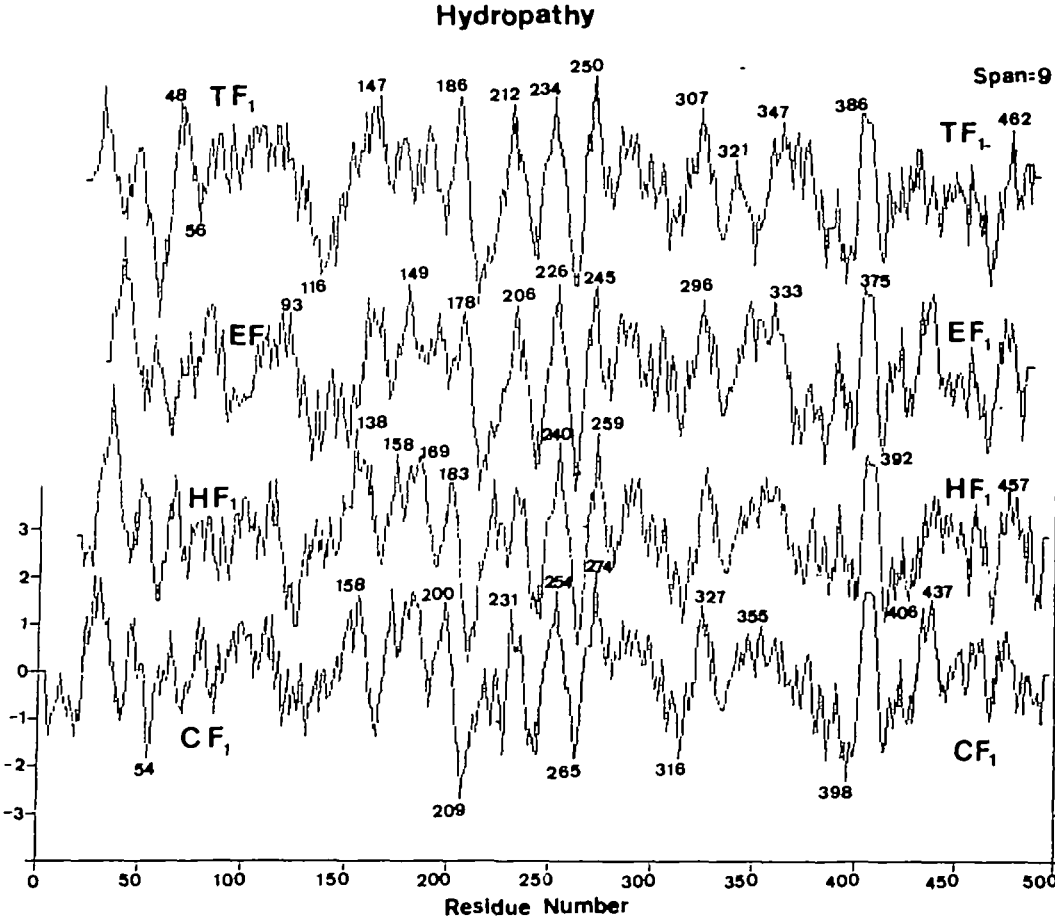

Fig. 5. Distribution of hydrophilicity in the peptide sequences of β subunits of thermophilic F_1 (TF_1), *E. coli* F_1 (EF_1) chloroplast F_1 (CF_1), and human mitochondrial F_1 (HF_1). The curves were drawn by the method of Kyte and Doolittle (*47*) using a Genetyx program for an NEC PC9801 computer. The signal presequence of F_1 of human mitochondria has been removed.

TABLE III. Benefit of different residues in $TF_1\beta$ from those at homologous positions of $F_1\beta$'s of mesophiles for increasing stability.

Stabilizing effects	Different residues in $TF_1\beta$
Helix formation	(D43, D45)[a], L48, E332, E375, M469
Sheet formation	V6, V95, Q169, F408
Internal hydrophobicity	F215, V344, S426, R405, (A355)
External polarity[b]	(R3, K17, E19, E39, E41, D56, D57)
	D162, N423, K444, D451, R452, R454
Internal packing	(I7, I26), I32, (I62)
	(I103, I109), I275, (A186)
Unidentified benefit, neutral to neutral	M10, T211, M213, Y313, Q419
	V430, V434, V463,
polar to neutral	V348
polar to polar	D394

[a] Residues of inserted sequence or partially homologous regions are shown in parentheses. [b] Residues mainly at the reverse turns.

TABLE IV. Codon usage in the β subunit of thermophilic (TF_1), *E. coli* F_1 (EF_1), and human F_1 (HF_1).

First position	Second position															Third position
	U			C			A			G						
	TF_1	EF_1	HF_1	TF_1	EF_1	HF_1	TF_1	EF_1	HF_1	TF_1	EF_1	HF_1				
U	Phe	4	3	7	Ser	0	7	12	Tyr	5	4	7	Cys	0	1	0	U
U	Phe	13	11	8	Ser	4	14	3	Tyr	7	11	5	Cys	0	0	0	C
U	Leu	2	0	4	Ser	0	2	2	Term[a]	0	1	0	Term[a]	1	0	1	A
U	Leu	23	1	10	Ser	12	0	3	Term[a]	0	0	0	Trp	0	1	0	G
C	Leu	4	3	2	Pro	0	4	11	His	5	0	9	Arg	5	21	7	U
C	Leu	6	3	2	Pro	0	0	6	His	7	7	0	Arg	16	5	2	C
C	Leu	0	0	5	Pro	1	0	9	Gln	17	2	7	Arg	0	0	1	A
C	Leu	5	35	18	Pro	22	16	0	Gln	4	18	16	Arg	6	0	2	G
A	Ile	15	4	17	Thr	0	5	12	Asn	1	2	5	Ser	0	0	2	U
A	Ile	20	23	18	Thr	3	18	14	Asn	10	9	4	Ser	1	2	1	C
A	Ile	0	0	1	Thr	7	0	4	Lys	18	17	12	Arg	1	0	6	A
A	Met	14	15	13	Thr	19	1	1	Lys	2	3	11	Arg	0	0	3	G
G	Val	7	12	11	Ala	4	7	22	Asp	11	6	17	Gly	7	29	21	U
G	Val	22	6	6	Ala	16	6	16	Asp	18	21	9	Gly	25	14	14	C
G	Val	2	19	10	Ala	1	8	7	Glu	27	27	19	Gly	5	2	6	A
G	Val	11	10	16	Ala	18	13	2	Glu	14	12	17	Gly	6	0	5	G

[a] Term, terminus.

The stability and reconstitutability may be achieved by many small changes throughout the $TF_1\beta$ molecule without change in the homologous functioning sites. The differences in TF_1 residues compared with those of residues common to CF_1, HF_1, and EF_1 can be classified into five categories of benefits to stability (*22*) (Table III).

Codon Usage for the $TF_1\beta$ Subunit—Despite the high homology in amino acid sequences of F_1's, the codon usage for $TF_1\beta$ is quite different from those of $EF_1\beta$ (*4*, *5*) and $HF_1\beta$ (*28*) (Table IV). The codons for $CF_1\beta$ are also different because they are rich in adenine and uridine (*6–8*). The distribution curve of contents of guanine and cytosine plotted against residue numbers from the N-terminal was very different from those of CF_1, HF_1, and EF_1. The gene for EF_1 has a codon distribution typical of moderately to highly expressed *E. coli* genes (*48*). Except for Gln, Lys, and Glu, amino acids are coded by codons ending with G or C (Table III). Even these exceptions were not found in the codon usage of an extreme thermophile (*36*), perhaps to stabilize nucleic acid structure at high temperature. The results do not support the proposed idea (*22*) that only one base change per codon (*i.e.* minimal mutation) is needed to convert mesophilic proteins to thermophilic ones (Table IV).

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