Complete Amino Acid Sequence of Subunit e of Rat Liver Mitochondrial H^+-ATP Synthase[†,‡]

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Received August 31, 1992; Revised Manuscript Received October 8, 1992

ABSTRACT: Subunit e of H^+-ATP synthase from rat liver mitochondria was isolated from the purified enzyme by reverse-phase high-performance liquid chromatography. The amino acid sequence of the subunit was determined by automated Edman degradation of the whole protein and derived peptides. The nucleotide sequence of the import precursor of subunit e of rat liver H^+-ATP synthase was determined from a recombinant cDNA clone isolated by screening a rat hepatoma cell line H4TG cDNA library with a probe DNA. The sequence was composed of 289 nucleotides including a coding region for the import precursor of subunit e and noncoding regions on the 5′- and 3′-sides. The possible import precursor of subunit e and its mature polypeptide deduced from the open reading frame consisted of 71 and 70 amino acid residues with molecular weights of 8254 and 8123, respectively. Subunit e is a basic hydrophilic protein with an isoelectric point of 9.78. The sequence of the rat subunit e is highly homologous with that of subunit e of bovine heart, but has no homology with any subunit of bacterial or chloroplast H^+-ATP synthase. The function of subunit e is unknown. However, a homology search in the database of the National Biomedical Research Foundation revealed that residues 34–65 of subunit e are homologous with residues 90–117 of troponin T, and with residues 529–561 of h-caldesmon and residues 289–319 of l-caldesmon, which are the homologous sequences corresponding to the Ca^{2+}-dependent tropomyosin-binding region of troponin T.

Mitochondrial H^+-ATP synthase is a multisubunit complex that can utilize a transmembrane proton gradient to form ATP. H^+-ATP synthase is composed of two domains: a hydrophilic part, F_1, which is the catalytic site of ATP synthesis, and a membranous domain, F_o, which is responsible for energy transduction (Fillingame, 1980; Cross, 1981; Amzel & Pedersen, 1989; Hatefi, 1985, Futai et al., 1989).

F_1 in the mitochondria of mammals and yeast and in the membranes of chloroplasts and bacteria consists of five different polypeptides with the stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ (Walker et al., 1985). However, the δ- and ϵ-subunits of these F_1s are not identical: The δ- and ϵ-subunits of *Escherichia coli* are homologous to the oligomycin-sensitivity conferral protein and the δ-subunit of beef heart, respectively (Walker et al., 1985). Furthermore, the bovine (Walker et al., 1985) and rat (Higuti et al., 1992) ϵ-subunits are not related to any known subunits in bacterial and chloroplast ATP synthases. On the other hand, the subunit compositions of F_o are quite different in prokaryotes and eukaryotes. In *Escherichia coli*, F_o consists of only 3 different subunits (Fillingame, 1980), whereas mammalian mitochondrial H^+-ATP synthases are much more complicated: preparations of the enzyme isolated from bovine heart mitochondria have been reported to have 14 (Walker et al., 1991), 15 (Galante et al., 1981), or 16–18 (Ludwig et al., 1980) protein subunits, and preparations from rat liver 10 (McEnery et al., 1989) or 13–14 (Higuti et al., 1991; Yoshihara et al., 1991) subunits. It is unknown why mammalian H^+-ATP synthases are assembled from so many more polypeptide subunits than the prokaryotic H^+-ATP synthases.

Recently a new subunit component of mitochondrial F_o, named subunit e, was found in bovine heart (Walker et al., 1991) and rat liver (Cretin et al., 1991; Higuti et al., 1992) H^+-ATP synthase.

Here we report the primary structure of subunit e from rat liver, determined by direct analysis of the protein purified from rat liver H^+-ATP synthase and cDNA cloning.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (185 TBq/mmol) was from Amersham Corp., and various restriction endonucleases were from Takara Shuzo, Bethesda Research Laboratories, and New England Biolabs. Other chemicals used were reagent grade. A reverse-phase column of Daisopak SP 300_5C_{18} was donated from Daiso Co. (Osaka).

Purification of H^+-ATP Synthase and Subunit e. H^+-ATP synthase was purified to homogeneity from rat liver mitochondria as described previously (Yoshihara et al., 1991). Subunit e was purified from the purified rat H^+-ATP synthase as described previously (Higuti et al., 1992).

Cyanogen Bromide Cleavage of Subunit e and Purification of Peptide Fragments. The purified subunit e (50 μg) was dissolved in 50 μL of 70% formic acid, and cleaved by treatment with 50 μL of 70% formic acid containing 2.46 μmol of cyanogen bromide (about 400 times the amount of methionine in subunit e) for 24 h at about 25 °C. The fragments obtained were completely dried in a Tomy, Model cc-180, centrifuge concentrator and then dissolved in 50 μL of water containing 0.1% trifluoroacetic acid. The resulting solution was subjected to a reverse-phase column of Daisopak SP-1000_5C_{18} (4.6 × 250 mm) equilibrated with a solution containing 0.1% trifluoroacetic acid. Materials were eluted with a linear

[†] Supported by Grant-in-Aid for Scientific Research 04680196 (to T.H.), by Grant-in-Aid for Scientific Research on the Priority Areas of Cell Energy 04266215 (to T.H.), and by Grant-in-Aid for New Functionality Materials 02204009 (to Prof. Masuo Aizawa, Tokyo Institute of Technology) from the Ministry of Education, Science and Culture of Japan.

[‡] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under Accession Number D13121.

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0006-2960/92/0431-12451$03.00/0

gradient of a 0–100% solution of 80% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min at 40 °C.

Sequencing and Amino Acid Analysis of Subunit e. Automated sequence analyses of subunit e and its derived peptides were performed in an Applied Biosystems, Model 477A, protein sequencer equipped with a 120A phenylthiohydantoin derivative analyzer (Research Center for Protein Engineering, Institute for Protein Research). Amino acid analysis was carried out in a Waters PICO TAG high-performances liquid chromatography (HPLC)[1] system as described previously (Higuti et al., 1988).

Synthesis of Oligonucleotide Probe. Oligonucleotide hybridization probe was synthesized in an Applied Biosystems Model 380A synthesizer. 5′ end labeling of oligodeoxyribonucleotide with [γ-^{32}P]ATP and T4 polynucleotide kinase was carried out as described (Maxam & Gilbert, 1980).

Preparation of Poly(A)$^+$ RNA. Total RNA was extracted from H4TG (rat hepatoma cell line) by the guanidinium thiocyanate method (Chirgwin et al., 1979), and poly(A)$^+$ RNA was isolated using oligo(dT)–latex particles (Oligotex-dT30, Takara Shuzo).

Construction of a cDNA Library. A cDNA library of H4TG was constructed in a phagemid expression vector, Bluescript KS M13$^+$ (Stratagene). Double-stranded DNA complementary to H4TG poly(A)$^+$ RNA was synthesized as described (Fujiwara et al., 1989), except that 5′-TAGGTCGACGCGGCCGCTTTTTTTTTTTTTTT-3′ was used as primer for synthesis of the first strand of cDNA. This primer includes unique restriction endonuclease sites for *Not*I (5′-GCGGCCGC-3′) and *Sal*I (5′-GTCGAC-3′) at the 5′-side of $(dT)_{15}$. The double-stranded DNA mixtures synthesized were treated with Klenow fragment to blunt their ends. The resulting DNA mixtures were digested with *Not*I. Excess linkers and *Not*I-digested linker fragments were removed by fractionation on a QIAGEN-tip column (Funakoshi Co., Tokyo, Japan). The DNA mixtures were then ligated with vector Bluescript, which had been digested with *Not*I and *Eco*RV at the multicloning site. *Escherichia coli* HB101 competent cells (Takara Shuzo) were transformed with the ligated DNA.

Screening of the cDNA Library by Oligonucleotide Hybridization. For isolation of cDNAs for subunit e, about 150 000 transformants were screened by hybridization with ^{32}P-labeled oligodeoxyribonucleotide probe synthesized as described above. Colony hybridization was carried out by the method described (Hanahan & Meselson, 1980). Briefly, transformants that had been replicated onto duplicate nylon filters were prehybridized for 3 h at 42 °C in a solution of 5× SSC (44 g of NaCl and 22 g of sodium citrate in 1 L), 50 mM NaH_2PO_4, 50% formamide, 5× Denhaldt's solution, 1% SDS, and 250 μg/mL salmon sperm DNA and hybridized for 12 h in the same solution containing labeled probe (approximately 5×10^5 cpm/mL), which had a specific activity of about 6.5×10^6 cpm/pmol. The filters were washed sequentially with 2× SSC containing 0.1% SDS at room temperature for 15 min and at 42 °C for 15 min, and then exposed to X-ray film at −80 °C with an intensifying screen.

DNA Sequencing. DNA sequencing was carried out by the dye primer method in an Applied Biosystems, Model 373A, DNA sequencer.

Sequence Analysis of DNA and Protein. Sequence data were analyzed using the DNASIS program (Hitachi Software Engineering Co.) and the IDEAS program constructed by Dr. M. Kanehisa (Kyoto University).

[1] Abbreviations: HPLC, high-performance liquid chromatography; IF_1, ATPase inhibitor protein.

Table I: Amino Acid Compositions of Rat Subunit e and Derived Peptides

amino acid	intact protein	CB1	CB1′	CB2	CB2′	from sequence
Asp[a]	2.4	2.1	2.2			2
Thr	0.2	0.2				
Ser	4.2	2.1	2.0	2.5	2.2	4
Glu[b]	10.8	9.5	9.7	0.8	0.8	10
Pro	3.8	0.9	0.9	3.0	4.0	4
Gly	2.7	1.1	1.5	2.6	2.2	3
Ala	8.2	6.8	7.1	1.1	1.0	8
Val	5.1	1.4	1.1	3.5	3.3	4
Cys[c]						
Met	1.1					1
Ile	3.9	2.8	2.9	2.4	2.2	5
Leu	6.1	4.1	3.9	3.3	2.9	7
Tyr	4.5	3.0	3.1	1.9	1.2	4
Phe	2.2	1.2	0.9	1.1	1.4	2
Lys	8.1	6.2	6.3	0.8	0.5	7
His	0.3	0.1				
Arg	8.1	6.9	6.6	1.1	1.0	8
Trp	nd[d]	nd	nd	nd	nd	
residues		23–70	23–70	1–22	1–22	

[a] Sum of Asp and Asn. [b] Sum of Glu and Gln. [c] As cysteinic acid. [d] Not determined.

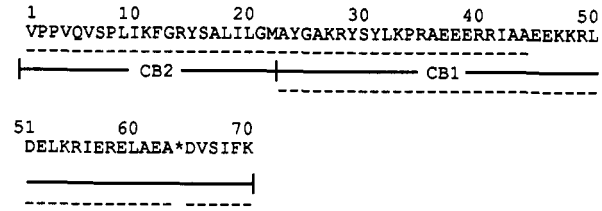
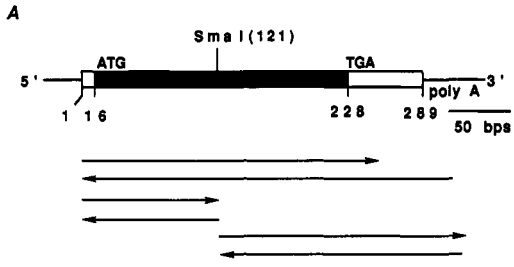


FIGURE 1: Determination of the amino acid sequence of subunit e of rat H$^+$-ATP synthase. Solid lines indicate the peptide fragments obtained by cleavage with cyanogen bromide. Dashed lines represent the sequences determined by Edman degradation.

RESULTS AND DISCUSSION

Amino Acid Sequence of Subunit e of Rat H$^+$-ATP Synthase. Subunit e of H$^+$-ATP synthase from rat liver mitochondria was isolated from the purified enzyme by reverse-phase HPLC as described previously (Higuti et al., 1992). Subunit e was cleaved with cyanogen bromide, and the fragments were separated by reverse-phase HPLC as described under Experimental Procedures. Four main peaks of fragment peptides were obtained and named CB1, CB1′, CB2, and CB2′ in order of their elution with a fifth peak of uncleaved subunit e (data not shown). The amino acid compositions of CB1 and CB2 were the same as those of CB1′ and CB2′, respectively (Table I). The primary structures of CB1 and CB2 were determined by direct analyses. Sequence and amino acid analyses of CB1 and CB2 revealed that these peptide fragments corresponded to the sequences of residues 23–70 and 1–22, respectively (Figure 1). The amino acid residue at position 64 was not determined by direct analysis.

Isolation of cDNA Clones Encoding Rat Subunit e. A rat cDNA library was constructed with the phagemid Bluescript vector using poly(A)$^+$ RNAs extracted from H4TG (hepatoma cell line) as described under Experimental Procedures. For the isolation of cDNA for subunit e, this library was screened by hybridization with synthetic deoxyribonucleotide as probe. The probe DNA was synthesized on the basis of the determined amino acid sequence of residues 38–49 of rat subunit e (Figure 1) and the sequence of cDNA for bovine subunit e (Walker



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1   GAGGTCACGGACAAAATGGTGCCCCCGGTTCAGGTCTCTCCGCTCATC   48
1                M  V  P  P  V  Q  V  S  P  L  I      11
                 |_____Mature rat subunit e_____|
49  AAGTTCGGCCGGTACTCCGCTCTGATCCTCGGCATGGCATACGGCGCC   96
12   K  F  G  R  Y  S  A  L  I  L  G  M  A  Y  G  A    27

97  AAGCGCTACAGTTACTTAAAACCCCGGGCAGAGGAGGAGAGAAGAATA  144
28   K  R  Y  S  Y  L  K  P  R  A  E  E  E  R  R  I    43

145 GCAGCGGAGGAAAAAAAGAGACTAGATGAGTTGAAACGGATTGAGAGA  192
44   A  A  E  E  K  K  R  L  D  E  L  K  R  I  E  R    59

193 GAACTGGCAGAAGCTGAAGATGTCAGCATTTTCAAGTGAGGCGGCAGT  240
60   E  L  A  E  A  E  D  V  S  I  F  K  *              71

241 GATCTTGCTTTTCTCTTGTCGTTGAGGATGAATAAAGCTTCATTGTGT  288
289 GAAAAAAAAAAA                                      300
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FIGURE 2: (A) Restriction endonuclease map of cloned cDNA for rat subunit e and the sequencing strategy. The solid and open boxes show the coding region for subunit e and 5′- and 3′-noncoding regions, respectively. Sequenced regions are shown by horizontal arrows. (B) Nucleotide sequence of the cDNA insert encoding subunit e of rat liver H+-ATP synthase and the amino acid sequence deduced from its open reading frame. Nucleotides are numbered in the 5′- to 3′-direction. The predicted amino acid sequences of subunit e and its possible presequence are shown below the nucleotide sequence. Amino acid residues are numbered from the N-terminus. Solid lines show the amino acid sequences determined by Edman degradation. The possible polyadenylation signal AATAAA is doubly underlined.

et al., 1991). Its sequence was as follows: 5′-GA(A/G)-GA(A/G)GA(A/G)AGGAGGATTGCAGCCGA(A/G)-GA(A/G)AA(A/G)AA-3′. We screened about 150 000 colonies of a H4TG cDNA library with the synthesized 35-mer mixed oligonucleotides, and isolated 3 cDNA clones that gave strongly positive signals for the probe DNA from the library by the colony hybridization technique. The sequence of 1 of these 3 clones, which had a cDNA insert of about 300 nucleotides length, was determined.

Primary Structure Deduced from the cDNA Sequence. The nucleotide sequence of the rat subunit e cDNA was determined by the strategy illustrated in Figure 2A. The resulting nucleotide sequence of the cDNA, the primary structure of the mature subunit e, and the possible presequence deduced from the cDNA sequence are shown in Figure 2B. The sequence of 289 nucleotides included the entire coding region and the 5′- and 3′-noncoding regions.

The 3′-noncoding region consisted of 63 nucleotides, excluding the poly(A) tail. A polyadenylation signal (AATAAA), which is common in eukaryotic mRNAs (Proudfoot & Brownlee, 1978; Wickens & Stephenson, 1984), was located 14 nucleotides upstream from the poly(A) addition site. Part of a poly(A) sequence (approximately 100 nucleotides) was seen at the 3′-end of this cDNA clone.

The mature protein-coding region is 210 nucleotides long, is capable of encoding a protein of 70 amino acids with a molecular weight of 8123, and extends from GTG at nucleotide positions 19–21 to nucleotide position 228. This coding sequence is followed by the termination codon TGA. The amino acid sequence (69 amino acid residues) determined by

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                  40          50          60
Subunit e:    KPRAEEERRI-AAEEKKRLD-ELKRIERELAEAE
                  :::::   :::::     : ::   : : :::
Troponin T:   RKKEEEEERRVREAEEKKQREIEEKRM-R-LEEAE
                 90        100         110

                  40          50          60
Subunit e:    KPRAEEERRI-AAEEKKRLDELKRIERELAEAED
                  :   : :: :   :::::  :   :::  :::
h-Caldesmon:  KKQEEAERKIREEEEKKRMKE-E-IERRRAEAAE
                       540         550         560

                  40          50          60
Subunit e:    KPRAEEERRI-AAEEKKRLDELKRIERELAEAED
                  :   : :: :   :::::  :   :::  :::
l-Caldesmon:  KKQEEAERKIREEEEKKRMKE-E-IERRRAEAAE
                       300         310         320
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FIGURE 3: Alignments of homologous sequence of rat subunit e, troponin T, h-caldesmon, and l-caldesmon. Homology search of subunit e in the databank of the National Biomedical Research Foundation was carried out using the IDEAS program constructed by Dr. M. Kanehisa (Kyoto University).

automated Edman degradation was found to be in complete accordance with that deduced from the cDNA sequence. The amino acid sequence of the mature rat subunit e deduced from the cDNA was highly homologous with that of the bovine subunit e (Walker et al., 1991), 58 of 70 amino acids being identical. However, it had no homology with the sequences of any of the subunits of bacterial or chloroplast H+-ATP synthase.

Only one ATG was found in the presequence of the coding region, as in the bovine subunit e. Thus, as the presequence of the rat subunit e could be one methionine residue, the import signal of the subunit e should be contained in its mature sequence.

Hydropathy and charge distribution plots of the amino acid sequence showed that the rat subunit e is a basic hydrophilic protein. The isoelectric point of the protein was calculated to be 9.78 from its amino acid composition.

Bovine (Walker et al., 1991) and rat (Higuti et al., 1992) mammalian H+-ATP synthases consist of at least 14 different polypeptides. There are no counterparts in *Escherichia coli* for six of these polypeptide subunits, namely, the mammalian ϵ-subunit in F_1 (Walker et al., 1985; Vinas et al., 1990; Higuti et al., 1992), coupling factor 6 (Fang et al., 1984; Walker et al., 1987a; Higuti et al., 1990, 1991), subunit d (Walker et al., 1987b; Motojima & Imanaka, 1992; Higuti et al., 1992), IF_1 (Frangione et al., 1981), chargerin II (A6L) (Fearnley & Walker, 1986; Uchida et al., 1987; Higuti et al., 1988; Oda et al., 1989; Muraguchi et al., 1990), and subunit e. The functions of these extra subunits are unknown, but one of the possible roles of the the extra subunits could be a regulatory one. Recently, Das and Harris reported the existence of a regulatory mechanism in normal heart acting directly at the level of H+-ATP synthase and showed that the capacity of this enzyme can be switched to a low or high value depending on energy demand, by means of a Ca^{2+}-dependent regulatory system [cf. Harris and Das (1991)]. In this connection, it is interesting that as shown in Figure 3, a homology search in the database of the National Biomedical Research Foundation revealed that residues 34–65 of the rat subunit e show homology with residues 90–117 of troponin T with identity of 18/34, and to residues 529–561 of h-caldesmon with identity of 17/34 and residues 289–319 of l-caldesmon with identity of 17/34, which are the homologous sequences corresponding to the Ca^{2+}-dependent tropomyosin-binding regions in troponin T (Bullard et al., 1988; Hayashi et al., 1991). Thus, subunit e could be involved in a Ca^{2+}-dependent regulatory system.

This could be one reason why the mitochondrial H^+-ATP synthase is assembled from a much greater number of different polypeptides than the prokaryotic one. However, extensive experiments are required to examine this possibility.

ACKNOWLEDGMENT

We thank Dr. Keiji Tanaka (Institute for Enzyme Research) for helpful discussion.

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