δ Subunit of Rat Liver Mitochondrial ATP Synthase: Molecular Description and Novel Insights into the Nature of Its Association with the F_1-Moiety[†,‡]

Weiying Pan, Young Hee Ko, and Peter L. Pedersen*

Department of Biological Chemistry, Johns Hopkins University, School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205-2185

Received January 9, 1998; Revised Manuscript Received February 20, 1998

ABSTRACT: The F_1 moiety of ATP synthase complexes consists of five subunit types in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\epsilon$. Of these, the δ subunit has received very little attention in the study of F_1 preparations from eukaryotic cells. Although recently shown to associate tightly with the β subunit [Pedersen, P. L., Hullihen, J., Bianchet, M., Amzel, L. M., and Lebowitz, M. S. (1995) *J. Biol. Chem. 270,* 1775−1784], the δ subunit is not resolved in the X-ray structure of either the rat liver or bovine heart enzyme. For these reasons, the novel studies reported here were designed both to provide a molecular description of the rat liver δ subunit and to gain insight into the nature of its interaction with F_1. The rat liver δ subunit was cloned from a λgt11 library, sequenced, overexpressed in *Escherichia coli* (*E. coli*) in fusion with the maltose binding protein, and, after cleavage of the latter protein, purified to homogeneity. The purified δ subunit (MW = 14.7 kDa) was shown by circular dichroism spectroscopy to be highly structured and to exhibit about 25% sequence identity to the chloroplast and *E. coli* ϵ subunits, frequently regarded as homologues of higher eukaryotic δ subunits. Significantly, and in contrast to the chloroplast and *E. coli* ϵ subunits, which are readily removed from their parent F_1 moieties after treatment respectively with ethanol and lauryldimethylamine oxide, the rat liver δ subunit remained tightly bound to F_1 under these relatively mild conditions. For the above reasons, four types of experiments were carried out on rat liver F_1 in order to (1) determine the accessibility of the δ subunit to both specific antibodies and to proteases, (2) establish the effect of nucleotides on this subunit's accessibility, (3) identify in cross-linking studies with disuccinimidyl glutarate this subunit's most reactive neighbor, and (4) determine whether this subunit can be dissociated from F_1 by using ionic detergents while leaving the remaining complex intact. The data derived from this detailed set of studies (a) supports the view that the rat liver F_1-δ subunit is in very close proximity to the γ subunit near the bottom of the F_1 molecule but does not penetrate deeply into the central core, (b) shows that within F_1 the δ subunit's N-terminus is exposed while its C-terminus is masked, (c) indicates that access to the δ subunit is shielded in part by the α, β, and γ subunits and changes during the catalytic cycle of F_1, and (d) implicates the δ subunit as important for the structural stability of the F_1 unit. These novel findings on a higher eukaryotic F_1-δ subunit are discussed in relationship to earlier studies on the related ϵ subunits from both chloroplasts and *E. coli*.

ATP synthase complexes represent some of the most important and fascinating membrane components in biological systems where they participate in oxidative or photosynthetic phosphorylation (*1*−*6*). These enzyme complexes are comprised of 8, 9, and 16 subunit types respectively in *Escherichia coli* (*E. coli*) (*4*), chloroplasts (*7*), and animal systems (*8*). Five subunit types, in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\epsilon$, comprise the water-soluble unit of the complex called F_1, whereas the remaining subunits are found in the detergent soluble unit called F_0.[1] F_1 is a catalytic unit which can bind and hydrolyze ATP but, when bound to F_0, can synthesize ATP at the expense of an electrochemical proton gradient. The overall ATP synthase complex (F_0F_1) has a tripartite structural appearance (*9, 10*) with the F_0 unit comprising predominantly a membrane sector, the F_1 unit comprising predominantly a spherical headpiece, and specific subunits or subunit parts from F_0 and F_1 comprising a stalk or "bridging unit".

The stalk region of ATP synthase complexes from *E. coli* and chloroplast has received considerable attention in studies designed to understand how the energy conserved within the electrochemical proton gradient is transduced to the F_1 unit. In particular, the ϵ subunit is believed to be a central player. Studies by Kuki et al. (*11*) first demonstrated the importance of the N-terminal region of the *E. coli* ϵ subunit for mem-

[†] This work was supported by NIH Grant CA 10951 to P.L.P.

[‡] GenBank Accession Number U00926.

* To whom correspondence should be addressed: Phone 410-955-3827. Fax 410-614-1944.

[1] Abbreviations: F_1, catalytic moiety of ATP synthase; CF_1, chloroplast F_1; EF_1, *E. coli* F_1; MBP, maltose binding protein; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DSG, disuccinimidyl glutarate; LDAO, lauryldimethylamine oxide; PBS, phosphate buffered saline; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; pfu, plaque forming unit; LB, Luria−Bertani; IPTG, isopropylthio-β-D-galactoside.

S0006-2960(98)00069-5 CCC: $15.00
Published on Web 04/22/1998

brane binding of F_1. Subsequent studies with the same enzyme clearly demonstrate that its C-terminal region interacts with the F_1 $\alpha_3\beta_3$ subdomains (*12*−*15*) and its N-terminal region interacts with the c subunit of F_0 (*16*). In addition, ϵ has been shown to cross-link or interact with the γ subunit (*17*, *18*) and to form a 1:1 complex in solution (*17*). Significantly, recent structural studies using NMR reveal that the *E. coli* ϵ subunit is organized in two domains, a 10 stranded β sandwich structure at the N-terminus, which interacts with the c subunit of F_0 and an α helix/loop/helix structure at the C-terminus, which interacts with the $\alpha_3\beta_3$ subdomain (*19*). This latter interaction is nucleotide dependent with the ϵ's interaction with the β subunit's C-terminal region (*20*) being favored in the presence of ADP and its interaction with the α subunit's C-terminal region being favored in the presence of ATP (*12*−*15*). This dynamic behavior of the ϵ subunit in *E. coli*, which gained its earliest convincing support from electron microscopy (*21*), and more recent support from biochemical (*22*) and biophysical studies (*23*), led to the view that significant movements of one or more small subunits (γ, δ, or ϵ) may be essential for the participation of all three β subunits in catalysis. This view has now gained considerable momentum following the recent experiment demonstrating that ATP hydrolysis can, in fact, induce physical rotation of the γ subunit in F_1 immobilized on a solid surface (*24*, *25*).

In addition to their role in energy coupling, the ϵ subunits of the *E. coli* and chloroplast F_1 units are known to suppress ATP hydrolysis, which can be activated following their dissociation from F_1. Significantly, dissociation can be readily achieved for the *E. coli* enzyme either by using a monoclonal anti-ϵ affinity column (*26*) or by using the detergent LDAO (*27*) and for the chloroplast enzyme by addition of ethanol (*28*). As expected, both the *E. coli* and chloroplast ϵ subunits inhibit the ATPase activity of the ϵ-depleted F_1 preparations (*27*−*29*).

The δ subunit of animal ATP synthases is frequently considered to be structurally and functionally equivalent to the ϵ subunit of the *E. coli* and chloroplast enzymes. However, in animal systems an extra subunit called IF_1, which is readily bound and released from F_1, serves as an ATPase inhibitor (*30*, *31*). In addition, animal ATP synthases contain many more F_0 subunit types (*7*, *8*) than those found in the *E. coli* and chloroplast enzymes (*4*, *7*). Therefore, it remains possible that δ subunits of animal ATP synthases, although playing in part roles similar to ϵ subunits of the bacterial and plant enzymes, also play distinct roles as well. Unfortunately, insight into this issue has not been resolved to date by X-ray crystallography, as high-resolution structures have not been obtained for either the complete *E. coli* or chloroplasts enzymes (i.e., F_0F_1), nor has such a structure been obtained for animal F_0F_1 ATP synthases. Although structures at 3.6 and 2.8 Å resolution have been reported for the rat liver and bovine heart F_1 units, respectively (*32*, *33*), the δ subunit is not resolved in either case.

For the above reasons, the novel studies described here were designed both to provide a molecular description of the δ subunit from an animal system, in this case rat liver, and to gain insight into the nature of this subunit's interaction with the F_1 unit. In the absence of such information from recent X-ray structures of F_1 (*32*, *33*), the information acquired from the experiments described below is fundamental to our eventual understanding of ATP synthase structure and function in eukaryotic cells.

EXPERIMENTAL PROCEDURES

Materials

Rats (Sprague-Dawley, white males) were obtained from Charles River Breeding Laboratories. The Lambda gt11 rat liver cDNA library and Y1090r *E. coli* cells were obtained from Clontech. Nitrocellulose filters were from Schleicher and Schuell, and LB medium was from Difco Laboratories. [γ-^{32}P]dATP was from New England Nuclear. DH5α *E. coli* cells were from Life Technologies. Polynucleotide kinase, restriction enzymes, DNA ligase, and the protein fusion and overexpression kit were obtained from New England Biolabs. Calf intestinal alkaline phosphatase was from Boehinger Mannheim and Sequenase from United States Biochemicals. GeneAmp PCR reagent kit with AmpliTaq DNA polymerase was from Perkin-Elmer. Oligonucleotides and peptides were synthesized in the Protein/Peptide/DNA Facility, Johns Hopkins University. Factor Xa, the cross-linking agent disuccinimidyl glutarate (DSG), dimethylpimelimidate, and the bicinchoninic assay kit for determining protein were from Pierce. ATP, ADP, AMP−PNP, Tris, $MgCl_2$, EDTA, pyruvate kinase, lactic dehydrogenase, bovine serum albumin (fraction V), and carboxypeptidase Y were from Sigma. Modified, sequencing grade trypsin was from Boehringer Mannheim Biochemicals. Electrophoresis grade chemicals, the Prep-A-Gene kit, PVDF (polyvinylidene difluoride) membranes for protein sequencing, Affi-Gel Protein A Agarose, and the Coomassie dye binding assay kit for determining protein were from Bio-Rad. The enhanced chemiluminescence (ECL) detection kit, rainbow molecular weight markers, the antimouse IgG-horseradish peroxidase-linked whole antibody (sheep), the antirabbit IgG-horseradish peroxidase-linked antibody (donkey), and detection solution were from Amersham. A monoclonal antibody (mAb195) specific for residues 1−16 of the bovine heart F_1-δ subunit was generously provided by Professor Joel Lunardi, Université Joseph Fourier, Grenoble, France, and prepared as previously described (*34*). Immulon microtiter plates were from Dynatec. Chloroplast F_1-ATPase (CF_1) was generously provided by Dr. Richard McCarty, Department of Biology, Johns Hopkins University. The Protein Pak 125 HPLC gel filtration column was from Waters-Millipore Corp. Sephadex G-50 was from Pharmacia Biotech Inc. and the concentrating system of Centricon and Microcon from Amicon. Phosphate buffered saline was from Gibco, and sodium phosphate and potassium phosphate were from J. T. Baker Chemical Co. All other agents were of the highest purity commercially available.

Methods

Library Screening for the cDNA Clones of the Rat Liver F_1-δ Subunit. A rat liver cDNA library was screened by using a [γ-^{32}P]dATP-labeled oligonucleotide probe, which was synthesized based on the bovine heart F_1-δ sequence encoding amino acids Gln 35 through Val 65. The transfer of γ^{32}P from [γ-^{32}P]dATP to the 5′-terminus of the probe was catalyzed by polynucleotide kinase. Y1090r *E. coli* cells were used as the host cell line for the cDNA library. During

the first round of screening 30,000 pfu per plate were screened. Duplicate nitrocellulose filters were lifted from each plate and the DNA was then denatured and baked onto the filters according to the instructions of the library manufacturer (Clontech). Filters were prehybridized in 0.09 M sodium citrate, pH 7.0, 0.9 M NaCl, 0.04% ficoll, 0.04% polyvinylpyrrolidone, 0.04% BSA, 0.25% SDS, 100 μg/mL denatured salmon sperm DNA for 3 h at 68 °C. Labeled probe was then added to this buffer and allowed to hybridize >12 h at 68 °C. After hybridization the filters were washed with 0.075 M sodium citrate, pH 7.0, 0.75 M NaCl, 0.1% SDS two times for 15 min at 25 °C. This was followed by a third wash with the same buffer for 30 min at 68 °C. Filters were air-dried and subjected to autoradiography. Positive plaques were selected and rescreened twice to isolate single clones. Second and third round screening followed the same protocol. Six positive clones were isolated.

Subcloning. λ phage DNA was purified from positive clones and digested with the restriction enzymes *Kpn*I and *Sac*I. Then the digested fragment which contained the F_1-δ gene was ligated into the plasmid pUC 19 which had been digested also with *Kpn*I and *Sac*I. The ligation products were transferred to DH5α *E. coli* cells.

DNA Sequencing. Double-stranded sequencing templates were isolated from transformed DH5α *E. coli* cells by using an alkali/SDS lysis procedure, which included precipitation with polyethylene glycol (*35*). The DNA was denatured with NaOH and then precipitated with ethanol. Sequencing was carried out using the dideoxy chain termination procedure of Sanger (*36*) in the presence of [α-^{35}S]dATP. Initially, λgt11 primers were used. Subsequent primers were constructed based on the resultant sequence, and this procedure was reiterated until both strands of the entire clone were completely sequenced.

Construction of the F_1-δ Subunit Expression Plasmid. The polymerase chain reaction (PCR) was used to generate the F_1-δ clone from the pUC 19 plasmid containing the cDNA encoding the δ subunit. Restriction enzyme sites *Fsp*I was incorporated into the forward primer, and *Hind*III and stop codon sites (TGA instead of TAG) were incorporated into the reverse primer:

forward primer: 5′-CCCGCGCGTGCGCATCACCTG-CCCCCGCC-3′

reverse pimer: 5′-GCGCGCGCGAAGCTTCACTCCA-GGGCCTTCACCAG-3′

PCR was carried out in a thermocycler (Coy Laboratories) for 30 cycles (94 °C:60 °C:72 °C − 1′;1′;2′), using 2.5 units of AmpliTaq DNA-polymerase in a 100 μL reaction mixture. The reaction conditions recommended by the enzyme's manufacturer (Perkin-Elmer) were followed. The PCR product was purified by using the Prep-A-Gene kit and cut with *Fsp*I and *Hind*III. The resultant DNA was resolved on a 1% agarose gel, isolated, and purified by using the Prep-A-Gene kit.

The expression vector pMAL-c2 was cut with *Xmn*I and *Hind*III. The restriction enzyme cut plasmid was isolated on a 1% agarose gel and purified by using the Prep-A-Gene kit. The F_1-δ subunit cDNA was ligated into the plasmid with T4 DNA ligase in frame with MBP and transformed into DH5α *E. coli* cells. Transformants containing insert DNA in the pMAL-c2 plasmid were selected on LB ampicillin agar plates by using the lacZ α-complementation assay. The plasmid was purified from the resultant colonies and sequenced by the method of Sanger *et* al. (*36*).

Overexpression and Purification of the MBP-δ Fusion Protein. DH5α *E. coli* cells containing the pMAL-c2-δ expression plasmid were grown to 2×10^8 cells/mL (A_{600} = ∼0.5) in 1 L of LB medium containing 0.2% glucose and 100 μg/mL ampicillin. Cells were induced with 0.3 mM IPTG and grown for 5 h at 37 °C and then harvested by centrifugation at 4000*g* for 20 min. The resultant cell pellets were resuspended in a 50 mL column buffer (20 mM Tris-Cl pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol) and frozen in a dry ice−ethanol bath. The sample was thawed in cold water and sonicated on ice in a Branson Sonifier cell disrupter at a power setting of 50 for a total of 6 min in 15-s pulses. The membrane fraction was removed by centrifugation at 9000*g* for 30 min at 4 °C. The supernatant was diluted with column buffer to ∼2.5 mg/mL and loaded onto a column containing amylose resin that had been previously equilibrated with the column buffer. The column was washed with 12 volumes of column buffer, after which the fusion protein MBP-δ was eluted with column buffer + 10 mM maltose and collected in 3-mL fractions. Protein was monitored by using the micro Coomassie dye binding assay kit from Bio-Rad, and the fractions containing protein were pooled.

Cleavage of the Fusion Protein and Purification of the F_1-δ Subunit. The isolated MBP-δ protein was cleaved specifically at the fusion junction with Factor Xa protease. The cleavage reaction was allowed to proceed at room temperature for >12 h at a 1/200 (w/w protease to protein) ratio. The cleavage mixture was loaded onto a hydroxyapatite column previously equilibrated with the column buffer, and the maltose in the cleavage mixture was then removed with 10 volumes of column buffer. The cleavage mixture was eluted with 0.5 M NaP_i, pH 7.2, in 2-mL fractions. Fractions containing the cleaved protein, detected by the Coomassie dye binding assay, were pooled and loaded onto an amylose column, and the flow-through was collected in 5-mL fractions. The protein in the flow-through, which was monitored by using the bicinchoninic assay kit of Pierce, consisted primarily of the δ subunit. The fractions containing the protein were pooled and concentrated by using a Centricon-3 concentrator and further purified by gel filtration HPLC (Protein Pak 125 column) in 0.1 M KP_i, pH 7.0. The δ subunit was eluted as a single peak, which was collected and subjected to SDS−PAGE followed by N-terminal sequence analysis.

N-Terminal Sequence Analysis. F_1 (150 μg) was separated by SDS−PAGE and was electrophoretically blotted onto a PVDF membrane. The δ subunit band was excised and subjected to degradation chemistry (*37*, *38*) using an Applied Biosystems model 475 A protein sequencing system, which employed both a gas and pulsed liquid phase.

C-Terminal Accessibility Analysis. F_1 (1.7 mg/mL) and the δ subunit (0.45 mg/mL) were incubated with 10 and 30 units, respectively, of carboxypeptidase Y for various times up to 4.5 h at 25 °C in 100 mM KP_i, pH 7.0. The reaction was quenched by addition of SDS−PAGE sample buffer and boiled for 5 min. Samples were subjected to SDS−PAGE and transferred electrophoretically to PVDF membranes.

Western blot analysis was then performed by using a polyclonal antibody (JH110) to the δ subunit with specificity for the C-terminal region (Glu 132 through Glu 142).

Circular Dichroism Spectroscopy. CD spectra were obtained at 25 °C in a 2 mm path length cuvette on an Aviv 60DS spectropolarimeter with the δ subunit at 2.7 μM in a 400 μL system containing 100 mM KP_i, pH 7.0. Spectra were deconvoluted by using the PROSEC program v2.1, which employs the reference spectra and algorithm of Chang et al. (*39*).

Peptide Synthesis and Antibody Production. Peptide segments of the F_1-δ subunit were synthesized on an Applied Biosystems model 430 A peptide synthesizer using the solid-phase method (*40*). Two peptides were synthesized, one called JH60 corresponding to the region from His 47 through Thr 69 of the bovine F_1-δ subunit and one called JH110 corresponding to the last 11 amino acids of the C-terminal region (Glu 132 through Glu 142) of the rat liver δ subunit. Both peptides were conjugated to BSA (fraction V) after which polyclonal antibodies were raised in rabbits by Spring Valley Laboratories (Woodbine, MD). The adjuvant for the first injections was Freund's complete adjuvant, whereas subsequent injections were in Freund's incomplete adjuvant. An antibody to the entire F_1-δ subunit called JH142 was raised by the same procedure.

Preparation of the Anti-δ Subunit Protein A Agarose Beads and Binding of the δ Subunit. The anti-δ subunit serum (1 mL) was diluted 1-fold with PBS and added to 1 mL of protein A agarose beads previously equilibrated with PBS. The mixture was incubated at 25 °C for 1 h with gentle rocking, after which the beads were washed twice with 10 volumes of 0.2 M sodium borate (pH 9.0) followed by centrifugation at 10000*g* for 30 s, before suspending in 10 volumes of 0.2 M sodium borate (pH 9.0). Solid dimethyl pimelimidate was added to the mixture to give a final concentration of 20 mM. Then the coupling reaction was allowed to proceed for 30 min at 25 °C on a rocker and terminated by washing the beads once in 0.2 M ethanolamine (pH 8.0). The beads were then incubated for 2 h at 25 °C in 0.2 M ethanolamine with gentle mixing. The coupled beads were washed three times with 10 volumes of PBS and stored in PBS with 0.01% merthiolate at 4 °C.

Binding assays were carried out by incubating the purified recombinant δ subunit or F_1 with the coupled anti-δ subunit protein A beads at 25 °C on a rocker for 1 h after which the beads were centrifuged at 10,000*g* for 10 s. The supernatant was carefully removed and the resultant beads were washed three times with buffer. The SDS−PAGE gel sample buffer was added to the washed beads and subjected to boiling for 5 min to elute bound protein, after which the protein was analyzed by SDS−PAGE.

Enzyme-Linked Immunosorbant Assay (ELISA). The monoclonal antibody (mAb195) to the N-terminal region of the δ subunit was used to coat each well of immulon microtiter plates. To facilitate binding of the antibody (500 ng in 50 μL) to the wells, incubation was performed at 4 °C overnight, and then washing was done with 200 μL of PBS per well. Blocking of nonspecific sites in the wells was then achieved with 3% BSA in PBS (blocking buffer) for 1 h at 25 °C followed by washing four times with PBS. Varying amounts of δ subunit (0.5−128 ng) or F_1 (5−1280 ng) were then added to the microtiter plates already coated with mAb195. Subsequently, to the mAb195-δ conjugated system was added the JH142 polyclonal antibody (1/1000 dilution) and to the mAb195-F_1 conjugated system was added the polyclonal antibody against the whole F_1 (1/100 dilution). Dilution of the antibodies was in blocking buffer. Incubation was then carried out for 1 h at 25 °C. After the plates were washed four times with PBS, antirabbit IgG-horseradish peroxidase-linked whole antibody (sheep) was added at a dilution of 1/2000 in blocking buffer; this was then incubated 1 h at 25 °C and washed 4 times with PBS. Detection solution (100 μL), containing 50 mM sodium citrate, 50 mM citric acid, 0.1% *o*-phenylenediamine dihydrochloride, and 0.006% hydrogen peroxide, was then added to each well until the color became yellow, after which 50 μL of 2 M sulfuric acid was added to each well to stop the reaction. The absorbance was then read at 492 nm.

Purification of Rat Liver F_1-ATPase. The enzyme was purified by the procedure of Catterall and Pedersen (*41*) with the modification described by Pedersen et al. (*42*). The purified enzyme, in 250 mM KP_i and 5.0 mM EDTA, pH 7.5, was divided into 100 μL aliquots and lyophilized to dryness and stored at −20 °C until use.

Assay for ATPase Activity. ATPase activity was determined spectrophotometrically at 340 nm by coupling ADP production to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase (*43*), exactly as previously described for rat liver F_1 (*41*).

Gel Electrophoresis and Western Blotting. SDS−PAGE was carried out by the methods of Laemmli (*44*) using 1.0 mm, 15% polyacrylamide gels. Gels were either stained with Coomassie brilliant blue or transferred to PVDF membranes for Western blotting. Blots were blocked with 5% dry milk in PBS-0.2% Tween-20 and probed with a 1:5000 dilution of anti-δ antisera. Then detection followed the Amersham manufacturer's instructions and employed the enhanced chemiluminescence kit. Horseradish peroxidase-linked anti-rabbit IgG, used in a 1:10 000 dilution, was used as the secondary antibody.

Trypsin Digestion. F_1 (1.95 mg/mL) was dissolved in 250 mM KP_i and 5.0 mM EDTA, pH 7.5, and passed through a Sephadex G-50 spin column previously equilibrated with 50 mM NaP_i, pH 7.2. The resultant F_1 was diluted 1-fold with one of the following buffers: 50 mM NaP_i, pH 7.2; 50 mM NaP_i + 10 mM ATP + 2 mM EDTA, pH 7.2; 50 mM NaP_i + 10 mM $MgCl_2$ + 10 mM ATP, pH 7.2; 50 mM NaP_i + 10 mM $MgCl_2$ + 10 mM AMP·PNP + 10 mM ADP, pH 7.2. Samples were allowed to incubate for 30 min at 25 °C before addition of trypsin (1:16 w/w protease to protein). The cleavage experiments were performed at 37 °C. At various times, aliquots were withdrawn and trypsin activity was inhibited by PMSF (5 mM). The tryptic fragments were identified by SDS−PAGE (*44*) followed by Western blotting using an anti-δ antibody (JH142).

Cross-linking Experiments with Disuccinimidyl Glutarate (DSG). F_1 in 250 mM KP_i, pH 7.5, + 5 mM EDTA was pretreated by centrifugation in a tube containing a Centricon 100 membrane to remove the KP_i buffer and EDTA. The enzyme was resuspended in 50 mM NaP_i buffer, pH 7.2, for use in the cross-linking studies. Cross-linking experiments were carried out at 37 °C in a 100 μL system containing 47.5 mM NaP_i, pH 7.2, 60 μg of F_1, 5 μL of DMSO, and concentrations of DSG (dissolved in DMSO)

as indicated in the figure legend. Following the cross-linking assay, the mixture was subjected to SDS−PAGE and Western blot analysis using anti-δ antibody (JH142) exactly as described above under *Gel Electrophoresis and Western Blotting*.

Removal of the ϵ Subunit from CF_1. The ϵ subunit was removed from CF_1 (257 μg) essentially as described by Richter et al. (*28*) after prior treatment in a buffer (25 mM Tris-Cl, 0.5 mM ATP) containing 50 mM DTT followed by chromatography at 25 °C on a DEAE cellulose column (1.5 cm × 1 cm) in a cold buffer (previously on ice) containing 20% ethanol (v/v), 30% glycerol (v/v), 25 mM Tris-Cl, 0.5 mM ATP, and 5 mM DTT, pH 8.0. The latter buffer elutes the ϵ subunit while the $CF_1(-\epsilon)$ remains on the column: $CF_1(-\epsilon)$ is removed after the first washing with 25 mM TrisCl, 0.5 mM ATP, pH 8.0, to remove ethanol and glycerol and then with 25 mM TrisCl, 0.5 mM ATP, 0.4 M NaCl, pH 8.0.

Removal of the δ Subunit from Rat Liver F_1. F_1 in 250 mM KP_i, 5 mM EDTA, pH 7.5, was first precipitated twice at 25 °C and redissolved in 50 mM NaP_i, 5 mM ATP, 1 mM EDTA, pH 7.2. Selective removal of the δ subunit occurred within 30 min at 37 °C in a 100 μL system containing 50 μg of F_1, 0.0125% SDS, 50 mM NaP_i, 5 mM ATP, and 1 mM EDTA, pH 7.2. Following incubation, the mixture was placed in a tube containing a Microcon 100 membrane and subjected to centrifugation for 3 min in an Eppendorf microcentrifuge. The flow-through containing the δ subunit was collected and the fraction ($\alpha_3\beta_3\gamma\epsilon$) retained in the filter was washed three times with a buffer containing 50 mM NaP_i, 5 mM ATP, and 1 mM EDTA, pH 7.2. The tube containing the Microcon 100 membrane was reversed and centrifuged at 2000*g* for 3 min. The flow-through protein fraction and the retentate protein fraction from the second centrifugation step were then subjected to SDS−PAGE (*44*) using ~20−30 μg of protein.

Protein Determinations. Protein concentration was determined either by the Micro Coomassie dye binding assay of Bio-Rad or by the bicinchoninic assay of Pierce. BSA was used as the standard protein in both assays.

RESULTS

The Rat Liver F_1-δ Subunit Is a 14.7-kDa Protein, the N-Terminal Region of Which Is Predicted to Contain Predominantly β-Sheet/Strand Secondary Structure and the C-Terminal Region Predominantly α-Helical Structure. As very little information was available about the δ subunit of eukaryotic ATP synthases, experiments were undertaken first to clone, sequence, purify, and characterize the rat liver protein. In experiments described in detail under Methods, a 93 bp oligonucleotide probe based on the bovine F_1-δ subunit sequence (Runswick *et al*., 1990) was synthesized and used to screen a rat liver cDNA library. During the first round of screening six putative clones were obtained and subjected to two more rounds of screening. The phage DNA was purified, cut by the restriction enzymes *Kpn*I and *Sac*I and subcloned into the pUC19 vector as described under Methods. Following sequencing, an open reading frame was identified within an 809 bp positive clone. The deduced amino acid sequence of the rat liver F_1-δ subunit consists of 142 amino acids corresponding to a molecular weight of 14.7 kDa (Figure 1A). The predicted N-terminus of the mature δ subunit was based on N-terminal sequencing (*37*, *38*) of the δ subunit isolated from SDS−PAGE gels of purified F_1. The sequence obtained was ASPAPAAGPGQ. From the amino acid sequence alignments presented in Figure 2B, it can be concluded that the rat liver F_1-δ subunit exhibits about 91% identity to the bovine F_1-δ subunit (*45*) but only about 25% identity to the chloroplast and *E. coli* ϵ subunits (*46*−*48*) and about 32% identity with the yeast δ subunit.

The F_1-δ subunit was overexpressed in *E. coli* in fusion with the maltose binding protein (MBP) as described under

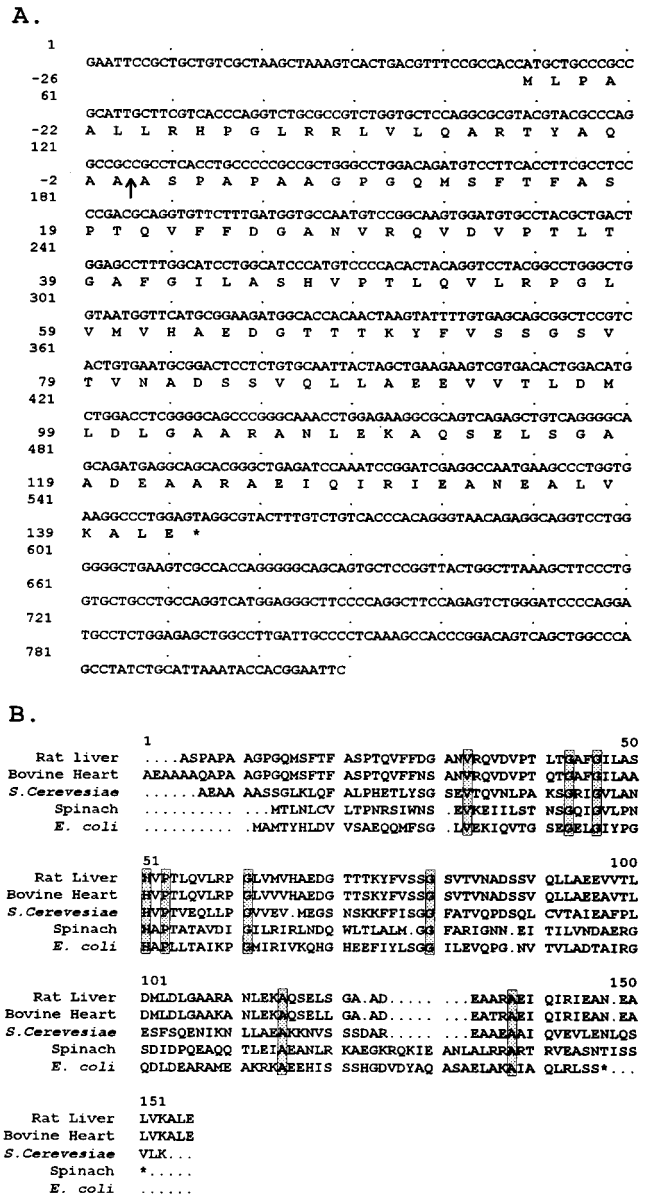

FIGURE 1: (A) cDNA and deduced amino acid sequences of the rat liver F_1-δ subunit. Cloning and sequencing of the rat liver F_1-δ subunit was carried out exactly as described under Methods. The complete 809 bp sequence and the deduced amino acid sequence (168 residues) are shown. The N-terminal starting point (arrow) of the mature δ subunit was determined by N-terminal sequencing of the δ subunit band excised from SDS−PAGE gels of purified rat liver F_1. (B) Comparison of the rat liver F_1-δ subunit amino acid sequence with that of bovine heart and yeast (*S. cerevesiae*) and with those of the ϵ subunit from F_1-ATPases present in *E. coli* and chloroplast (spinach). The regions designated with a shadow are the conserved residues among these sequences.

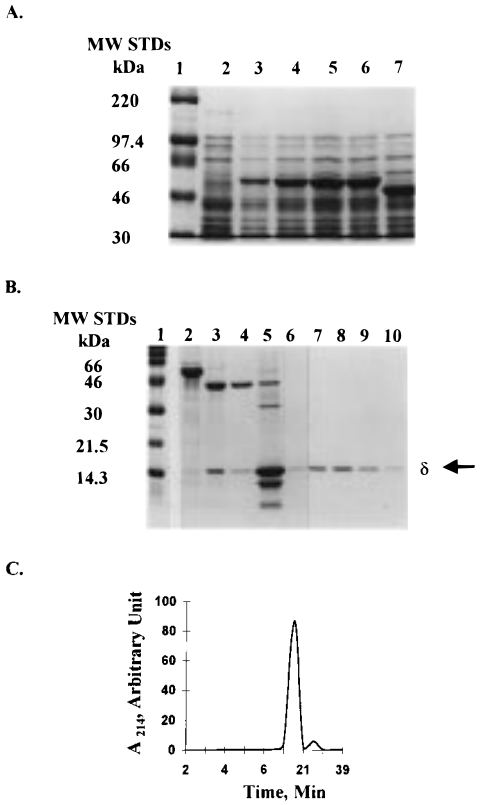

FIGURE 2: Overexpression and purification of the rat liver F_1-δ subunit. (A) SDS−PAGE gel of the MBP + δ fusion protein overexpressed in E. coli. Aliquots (25 μL) of the cell lysate were loaded onto SDS−PAGE gels (7.5%) and subjected to electrophoresis by the method of Laemmli (44). Proteins were then stained with Coomassie brilliant blue. Lane 1, rainbow high molecular weight markers; lane 2, uninduced cells; lanes 3, 4, 5, and 6, cells which contain the pMAL-c2-δ expression plasmid induced respectively for 1, 2, 3, and 5 h at 37 °C; lane 6, control cells which contain only the pMAL-c2 vector after induction for 4 h at 37 °C. (B) SDS−PAGE gels representative of different stages in the purification of the recombinant F_1-δ subunit. Purification of the F_1-δ subunit was carried out exactly as described under Methods. Aliquots representative of different stages of the purification were subjected to SDS−PAGE (15% polyacrylamide) by the method of Laemmli (44) and then stained with Coomassie brilliant blue. Lane 1, rainbow high molecular weight markers; lane 2, MBP + δ fusion protein after elution from an amylose column; lane 3, the fusion protein after cleavage with factor Xa; lane 4, the F_1-δ subunit after removal of maltose using a hydroxyapatite column; lane 5, flow-through fraction from the second amylose column; lanes 6, 7, 8, 9, and 10, different fractions within the F_1-δ subunit peak eluted from a Protein Pak 125 HPLC column (see Methods). (C) Typical HPLC profile of the purified recombinant F_1-δ subunit after elution in 0.1 M KP_i, pH 7.0 (see Methods).

Methods by using a pMal-C2 expression vector under control of the tac promoter (Figure 2A). The resultant MBP-δ fusion protein was then purified on an amylose column after which it was cleaved with Factor Xa and purified to apparent homogeneity by sequential steps involving chromatography on hydroxylapatite, amylose, and molecular sieve HPLC columns (Figure 2B,C). The secondary structure of the F_1-δ subunit, predicted from the program of Garnier et al. (*49*) (Figure 3A), and determined experimentally by circular dichroism (CD) spectroscopy (Figure 3B) of the purified recombinant subunit, consists of significant amounts of β-sheet/strand and α-helical character. In fact, the predicted structure (Figure 3A) indicates that the N-terminal half of

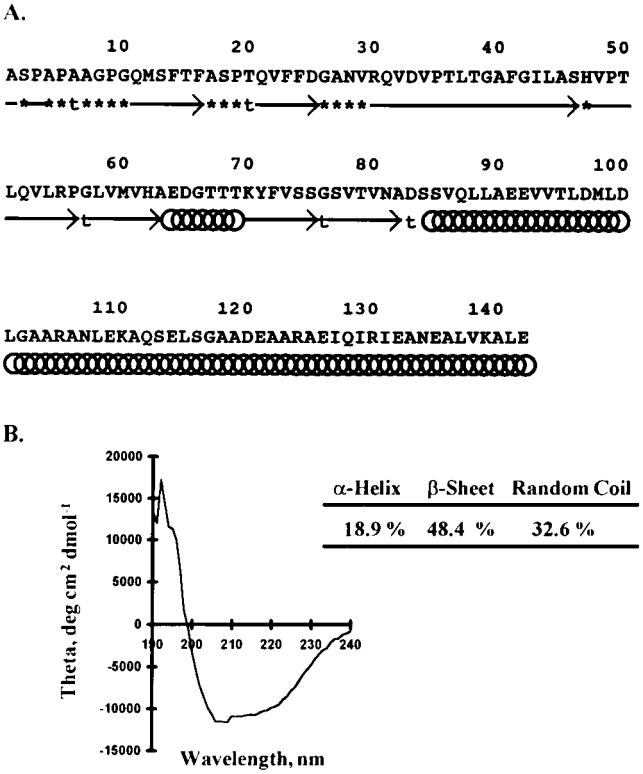

FIGURE 3: (A) Secondary structure prediction for the rat liver F_1-δ subunit. A graphical representation by the method of Garnier et al. (49) is presented. Coils depict helical structure; arrowheads depict extended or β-sheet structure, t depicts turns, and an asterisk depicts random coil. (B) Circular dichroism spectrum of the purified recombinant δ subunit. The CD spectrum and its deconvolution were obtained exactly as described under Methods.

the δ subunit is predominantly β-sheet/strand while the C-terminal half is predominantly α-helix. There is good agreement between the amount of β-sheet/strand structure predicted (∼40%) and that determined experimentally (∼48%), whereas there is more α-helical structure predicted (∼47%) than the ∼19% determined experimentally (compare parts A and B of Figure 3). The latter may result because the C-terminal region where the α-helical secondary structure is predicted to reside may not fully fold in the purified recombinant δ subunit because it requires interaction with its neighboring subunits within F_1.

The Rat Liver F_1-δ Subunit, in Contrast to the Partially Homologous ϵ Subunit of F_1 Preparations from Chloroplast and E. coli, Is Not Removed Respectively by Ethanol or by Lauryldimethylamine Oxide (LDAO). Although the δ subunit of F_1 preparations from both rat liver and bovine heart exhibit only about 25% identity with the ϵ subunits of the chloroplast and *E. coli* enzymes (Figure 1B), the latter subunits are frequently regarded as counterparts or "equivalents" of the higher eukaryotic δ subunit. As it has been well established that ethanol can dissociate the ϵ subunit from the chloroplast enzyme (*28*) and LDAO can dissociate the same subunit from the *E. coli* enzyme (*27*), the precise procedures used for these enzymes were applied to rat liver F_1. Results of control experiments shown in Figure 4A clearly demonstrate, as previously reported (*28*), that the ϵ subunit of chloroplast F_1 is readily removed following its chromatography on a DEAE cellulose column in a buffer containing 20% ethanol (see Methods). In sharp contrast, when the same procedure was

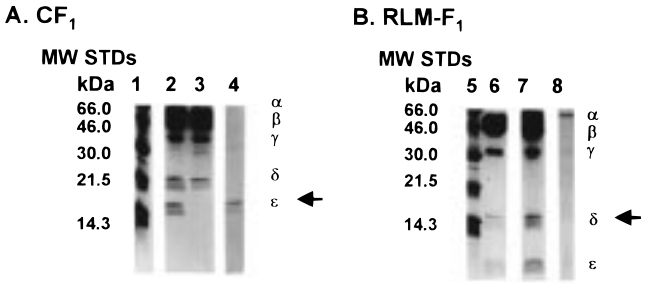

FIGURE 4: (A) SDS−PAGE gels of CF_1 before and after treatment with ethanol. The exact method used is described under Methods and started with 257 μg of CF_1 and employed a 20% ethanol buffered system and DEAE cellulose chromatography. Lane 1, rainbow high molecular weights markers; lane 2, CF_1 before ethanol treatment (control); lane 3, $CF_1(-\epsilon)$, which is the fraction bound to the DEAE−cellulose column; lane 4, ϵ, which is eluted with the buffer containing 20% ethanol. (B) SDS−PAGE gels of rat liver F_1 before and after treatment with ethanol. The procedure used was identical to that of CF_1. Lane 5, rainbow high molecular weight markers; lane 6, rat liver F_1 before ethanol treatment; lane 7, the fraction which bound to the DEAE cellulose column; lane 8, the fraction eluted with the buffer containing 20% ethanol.

applied to rat liver F_1, the δ subunit remained with the enzyme (Figure 4B). Also, in experiments not presented here, LDAO failed to selectively dissociate the δ subunit from rat liver F_1 under conditions previously successful for dissociating the ϵ subunit from *E. coli* F_1 (*27*). This involved treating F_1 in 100 mM NaP_i, 1 mM ATP, 1 mM DTT, and 0.03% LDAO, pH 7.5, and carrying out centrifugation for 16 h at 45000*g* in a 10−40% sucrose gradient, 25 °C. These results implicate a tighter association of the rat liver δ subunit with its F_1 moiety than that of the ϵ subunits of chloroplasts and *E. coli* with their respective F_1 moieties.

Experiments with Specific δ Subunit Antibodies Indicate That, within the Purified Subunit, Both the N- and C-Terminal Regions Are Exposed, Whereas within Intact F_1 Only the N-Terminal Region Is Exposed. As the relatively mild conditions used to dissociate the ϵ subunit from the chloroplast and *E. coli* F_1 moieties failed to selectively dissociate the analogous δ subunit from rat liver F_1, experiments with specific antibodies were carried out to determine the accessibility of the δ subunit within this enzyme complex. These studies entailed first acquiring a set of antibodies specific for different regions of the rat liver δ subunit and then comparing accessibility of these regions in the purified recombinant δ subunit (control) with their accessibility in intact rat liver F_1. Three different polyclonal antibodies were employed, one (JH60) to the internal δ subunit region His 47 through Thr 69, a second (JH110) to the C-terminal region Glu 132 through Glu 142, and a third (JH142) to the whole δ subunit. In addition, a monoclonal antibody (mAb195) with specificity for the 16 N-terminal residues of the bovine heart δ subunit (*34*) was employed. The relative capacities of these antibodies to bind to the purified δ subunit and to the δ subunit within intact rat liver F_1 was then tested with special emphasis on establishing the orientation of the N- and C-terminal regions, of which there are several different possibilities.

Each of the polyclonal antibodies described above was shown in control studies involving Western blot analysis (see Methods) to cross-react with both the denatured recombinant δ subunit (Figure 5A) and with the δ subunit within denatured F_1 (Figure 5B). The relative capacities of the polyclonal antibodies to bind to the purified recombinant δ subunit and to the δ subunit in intact F_1 was then tested in assays using agarose beads (see Methods). In separate experiments beads were coupled to each δ subunit polyclonal antibody (JH60, JH110, or JH142), and the capacity of the purified δ subunit and intact F_1 to bind to the coupled beads was then tested. Significantly, beads containing each polyclonal antibody bound the recombinant δ subunit (Figure 5C), indicating that the C-terminal region, the internal stretch from His 47 through Thr 69, and perhaps the N-terminal region are exposed. In contrast, only the beads containing the JH142 antibody to the whole δ subunit bound the δ subunit within the intact F_1 (Figure 5D). As beads containing polyclonal antibodies with specificity for either the internal region of the δ subunit (JH60) or to the C-terminal region (JH110) failed to bind the δ subunit within the intact F_1, this suggested that the latter two regions are not accessible but the N-terminal region may be accessible. To determine whether the N-terminal region is accessible mAb195 with specificity for the 16 N-terminal residues of bovine heart F_1 (*34*), where 11 of the last 12 residues are identical to those of rat liver F_1 (Figure 1B), was employed. Using an ELISA assay (see Methods) to conserve on the limiting amounts of antibody available, this N-terminal antibody, which in control experiments cross-reacts with the purified rat liver δ subunit (Figure 5E), was shown also to cross-react with the δ subunit within the rat liver F_1 (Figure 5F). Significantly, in these experiments F_1 remained fully active and therefore structurally intact when immobilized in the assay system.

Taken together these studies indicate that within the purified recombinant rat liver δ subunit both the C-terminal and N-terminal regions are exposed, whereas when this subunit is present in intact F_1 both the internal and C-terminal regions are inaccessible while the N-terminal region is exposed.

Intact Rat Liver F_1, upon Treatment with Carboxypeptidase Y, Remains Fully Reactive with the C-terminal δ Subunit Antibody (JH110), Whereas the Purified δ Subunit Subjected to the Same Treatment Rapidly Loses Reactivity. Both intact rat liver F_1 and the purified recombinant δ subunit (control) were incubated for various time periods with carboxypeptidase Y (see Methods). Following quenching of the reaction, the samples were subjected to SDS−PAGE, transferred to PVDF membranes, and analyzed by Western blot analysis for cross-reactivity with the δ C-terminal subunit antibody (JH110) described above. The results presented in Figure 6A,B show that during the time course of the experiment (up to 4.5 h) the C-terminal region of the δ subunit in the intact rat liver F_1 remains fully reactive with the JH110 C-terminal antibody, whereas the purified recombinant δ subunit loses reactivity to the antibody within 15 min (Figure 6B, compare lanes 1 and 2).

These studies further indicate that the C-terminal region of the δ subunit in intact rat liver F_1 is not readily accessible.

Studies with Trypsin Show That Partial Degradation of the α, β, and γ Subunits of Rat Liver F_1 Precedes Degradation of the δ Subunit and That Nucleotides Inhibit Degradation of All Four Subunits. To obtain insight into those factors responsible for the inaccessibility of a significant portion of the δ subunit, experiments were carried out to assess the ability of trypsin to proteolytically degrade this subunit within the intact F_1 complex. For these experiments F_1 was

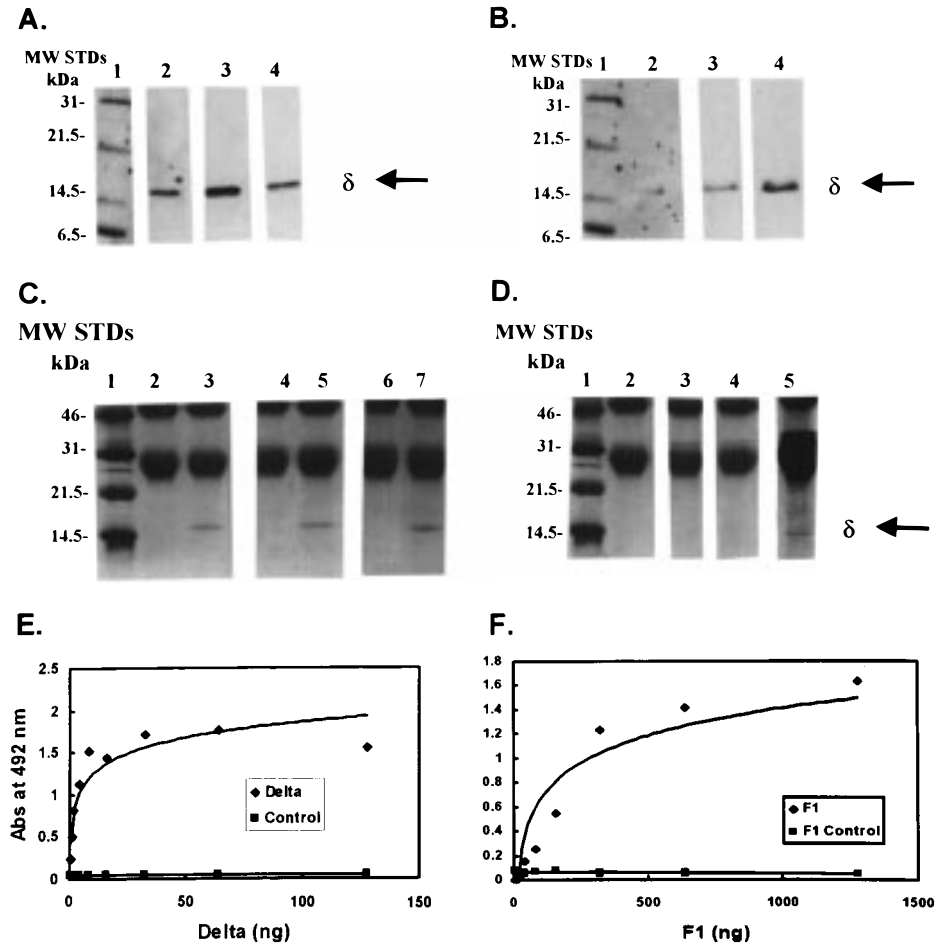

FIGURE 5: Relative accessibility of the recombinant δ subunit and the δ subunit within intact rat liver F_1 to anti-δ antibodies raised against different regions of the δ subunit. (A) Reaction of δ subunit polyclonal antibodies with the denatured δ subunit. Recombinant δ subunit (0.45 μg) was loaded onto 15% SDS−PAGE gels and subjected to electrophoresis. Proteins were transferred to PVDF membranes in 10 mM CAPS, pH 10 containing 10% methanol. In Western blot analysis (see Methods) the transferred proteins were then allowed to react with anti-δ antibodies. Lane 1, Biotinylated molecular weight markers; lanes 2, 3, and 4, interaction of the δ subunit respectively with JH60 (1/1000 dilution), JH110 (1/10 000 dilution), and JH142 (1/10 000 dilution). All dilutions were made in blocking buffer (5% dry milk and 0.2% tween 20 in PBS) after which the samples were allowed to incubate for 1 h. (B) Reaction of δ subunit polyclonal antibodies with the δ subunit in denatured F_1. Purified rat liver F_1 (5 μg) was loaded onto 15% SDS−PAGE gels and subjected to electrophoresis. Proteins were transferred to PVDF membranes as indicated in (A) and allowed to react with anti-δ antibodies. Lane 1, biotinylated molecular weight markers; lanes 2, 3, and 4, interaction of the δ subunit within denatured F_1 respectively with JH60 (1/1000 dilution), JH110 (1/10 000 dilution), and JH142 (1/10 000 dilution). All dilutions were made in blocking buffer (5% dry milk and 0.2% tween 20 in PBS) after which the samples were allowed to incubate for 1 h. (C) Capacity of the recombinant δ subunit to bind to anti-δ antibody-coupled protein A beads. Purified recombinant δ subunit (80 μL, 0.12 mg/mL) was incubated with 60 μL anti-δ antibody coupled A beads in 50 mM NaP_i, pH 7.2 at 25 °C on a rocker for 2 h. The beads were centrifuged at 10 000*g* for 10 s and the supernant was carefully removed. The resultant beads were washed three times with 50 mM NaP_i, pH 7.2, after which the bound proteins were eluted by boiling samples of beads in SDS−PAGE gel sample buffer for 5 min. The eluted proteins were loaded onto 15% SDS−PAGE gels and subjected to electrophoresis. Lane 1, rainbow high molecular weight markers; lanes 2, 4, and 6, JH60-, JH110-, and JH142-coupled protein A beads to which there was no added antigen (i.e., δ subunit); lanes 3, 5, and 7, JH60-, JH110-, and JH142-coupled protein A beads to which antigen (δ subunit) was added. (D) Capacity of the δ Subunit within the intact rat liver F_1 to bind to anti-δ antibody-coupled protein A beads. Purified, intact rat liver F_1 (80 μL, 0.35 mg/mL) was incubated with 60 μL anti-δ antibody-coupled A beads, washed, and centrifuged exactly as described in C. The bound proteins were eluted by boiling samples of beads in SDS−PAGE gel sample buffer for 5 min. The eluted proteins were loaded onto 15% SDS−PAGE gels and subjected to electrophoresis. Lane 1, rainbow high molecular weight markers; lane 2, JH142-coupled protein A beads to which there was added no antigen (i.e., intact rat liver F_1); lanes 3, 4, and 5, JH60-, JH110-, and JH142-coupled protein A beads to which antigen (intact F_1) was added. (E) Capacity of the purified recombinant δ subunit to bind to an anti-δ monoclonal antibody raised against the N-terminal region (ELISA assay). Monoclonal antibody (mAb195) (34) with specificity for the first 16 N-terminal residues of the bovine heart δ subunit, of which 11 of the last 12 are identical to those of rat liver F_1, was bound to Immulon microtiter plates. This was followed by addition of the purified recombinant δ subunit. To establish whether the δ subunit had bound to mAb195, the polyclonal δ subunit antibody (JH142) raised to the whole δ subunit was added followed by addition of the IgG-horseradish peroxidase color development system (see Methods for exact details). The exact system with the JH142 δ subunit antibody omitted served as a control. (F) Capacity of the δ subunit within intact rat liver F_1 to bind to an anti-δ monoclonal antibody raised against the N-terminal region (ELISA assay). Monoclonal antibody (mAb195) with specificity for the N-terminal region of the δ subunit (see Methods) was bound to Immulon microtiter plates. This was followed by addition of the purified, intact rat liver F_1. To establish whether F_1 had bound to mAb195, a polyclonal antibody to whole F_1 was added followed by addition of the antirabbit-IgG, horseradish perioxidase color development system (see Methods for exact details). The exact system with the anti-F_1 antibody omitted served as a control. The Y axis in this figure refers to absorbance at 492 nm as in (E).

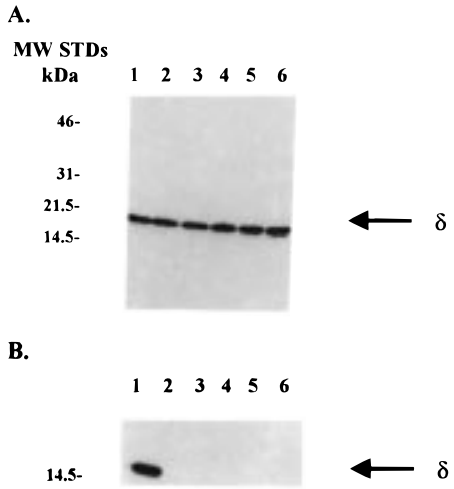

FIGURE 6: Accessibility of the C-terminal region of the rat liver δ subunit to carboxypeptidase Y when the subunit is within intact F_1 and when it is in isolated, purified form. Rat liver F_1 (A) or the purified, recombinant δ subunit from rat liver (B) was subjected to carboxypeptidase Y treatment exactly as indicated under Methods. After treatment, samples were quenched with SDS−PAGE sample buffer, subjected to SDS−PAGE, transferred to PVDF membranes, and then analyzed by Western blotting using a polyclonal antibody JH110 specific for the C-terminal region (Glu 132 through Glu 142). Lane 1, no carboxypeptidase Y (control); lanes 2−6, treatment with carboxypeptidase Y for 15 min, 30 min, 2-, 2.5 h, and 4.5 h respectively.

maintained in a NaP_i buffer, pH 7.2, and treated with trypsin over a time course that ranged from 10 min to 8 h, after which the reaction was terminated with the protease inhibitor PMSF. Control (untreated F_1) and treated samples were then analyzed by SDS−PAGE/Coomassie dye staining for protein bands and by Western blot analysis for the δ subunit.

Results obtained in the absence of nucleotides (Figure 7A,B) show that within 10 min the α and β subunits and the γ subunit have undergone degradation whereas the δ subunit is unaffected (compare lane 3 with control lanes 1 and 2). Within 30 min, however, degradation of the δ subunit commences (lane 4) and within 2 h it is no longer detectable even by Western blot analysis (lane 6). Results presented in Figure 7C,D show that, in the presence of ATP, degradation of the δ subunit and that of the α, β, and γ subunits is markedly reduced. In contrast, under catalytic conditions with ATP and Mg^{2+} present some degradation of the δ subunit, and of the α, β, and γ subunits, does occur (Figure 7E,F), but at a rate much less than in the absence of nucleotides (Figure 7A,B). As in the latter case, partial degradation of the α, β, and γ subunits precedes degradation of the δ subunit (compare lanes 3−5 with lanes 1 and 2). Finally, when both ADP and the nonhydrolyzable ATP analogue AMP·PNP are present with Mg^{2+}, there is little or no degradation of either the δ subunit or the α, β, and γ subunits.

These results indicate that partial degradation of the α, β, and γ subunits may be a prerequisite for degradation of the δ subunit, suggesting that this subunit is shielded by the larger subunits. These results also suggest that binding of nucleotides to α and β subunits promotes shielding of the δ subunit.

The Rat Liver F_1-δ Subunit Readily Cross-links with the γ Subunit in the Presence of a Low Concentration of Disuccinimidyl Glutarate (DSG). Studies with antibodies, carboxypeptidase Y, and trypsin described above indicated that much of the δ subunit is inaccessible in rat liver F_1 as a result of shielding by the larger subunits α, β, and γ. Previous studies (*50*) provided direct evidence that the δ subunit does interact with the β subunit, indicating that this subunit may be in part responsible for shielding the δ subunit. In an attempt to identify whether the α and γ subunits are near the δ subunit, rat liver F_1 was subjected to cross-linking studies. Although several cross-linking agents were tested the clearest results were obtained with DSG, a water soluble, homobifunctional *N*-hydroxysuccinimide ester with a spacer arm of 7.72 Å and a major specificity for the ϵ-amine of lysine. Coomassie stained SDS−PAGE gels presented in Figure 8A show, relative to control F_1 (lane 1), that as DSG concentration is increased from 0.0025 mM (lane 2) to 0.25 mM (lanes 6−8), the δ and the γ subunit bands lose staining intensity while a new band, δγ, is formed between the γ subunit band and the combined α + β band. Western blots of the same gel using a δ subunit antibody presented in Figure 8B show clearly the disappearance of the δ subunit band and the formation of the δγ band as DSG concentration is increased. In addition, N-terminal sequence analysis of the new band after transfer to PVDF membranes confirmed the presence of the rat liver γ subunit (sequence obtained = A T L K D I T). At concentrations of DSG higher than 0.25 mM, the δ and γ subunit bands and the new δγ band are almost lost (lane 10), indicating that higher molecular weight cross-linked products with the α and β subunits are formed which either remain at the top of the gel or fail to enter.

From these results, it seems clear that the γ subunit does reside very near the δ subunit in the intact rat liver F_1 complex, perhaps forming a δγ complex.

In the Presence of Low Concentrations of SDS the Rat Liver F_1-δ Subunit Can Be Selectively Removed from F_1 While Leaving the F_1 (-δ) Unit As an Inactive Complex. From the above experiments it seems clear that the δ subunit is tightly associated with the rat liver F_1 complex and that much of this subunit is shielded or "masked" by other F_1 subunits. The latter could result either from a deep penetration of the δ subunit into the central core of the F_1 molecule, perhaps together with the γ subunit, or from a close association with other F_1 subunits near the bottom of the molecule. If the δ subunit is integrated tightly into the core of F_1, a concentration of SDS capable of dissociating the whole complex would be required to solubilize the δ subunit. Conversely, if the δ subunit is localized near the bottom of the F_1 complex, low concentrations of SDS might result in its selective solubilization. To distinguish between these two possibilities, F_1 was incubated in a buffer containing ATP and varying concentrations of SDS and then placed in a tube containing a Microcon 100 membrane and centrifuged (see Methods). Subsequently, the protein that passed through the membrane (filtrate) and that retained on the membrane (retentate) were collected and subjected to SDS−PAGE and stained with Coomassie dye. As shown in Figure 9A, at the lowest concentration of SDS used, only the δ subunit of the five F_1 subunits is absent from the retentate (Compare lane 2, prior treatment with 0.0125% SDS, with lane 1, control F_1). Figure 9B (lane 2) shows that at this low concentration of SDS, the δ subunit is recovered in the filtrate with minor amounts of the α and β subunits. When higher concentrations of SDS are used there is a greater propensity

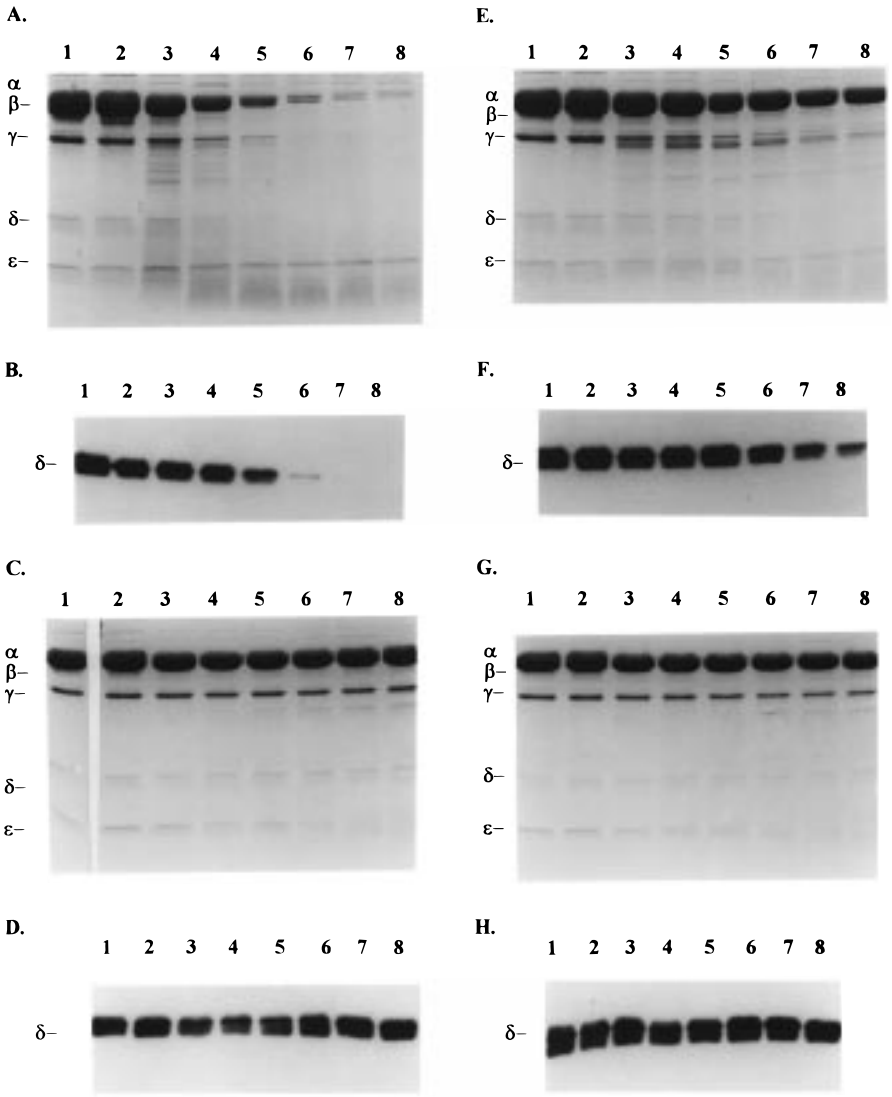

FIGURE 7: Accessibility of the δ subunit within intact rat liver F_1 to trypsin (see Methods for details). (A) Absence of nucleotides, SDS−PAGE gels. Lane 1, control F_1; lane 2, control F_1 incubated at 37 °C for 6 h without trypsin; lanes 3−8, F_1 treated with trypsin for 10 min and 0.5, 1, 2, 4, 6, and 8 h, respectively. (B) Absence of nucleotides, Western blot analysis in the region of the δ subunit. All conditions are the same as in (A). (C) Presence of 5 mM ATP and 1 mM EDTA, SDS−PAGE gels. Lane 1, control F_1; lane 2, control F_1 incubated at 37 °C for 6 h without trypsin; lanes 3−8, F_1 treated with trypsin for 0.5, 1, 2, 4, 6, and 8 h, respectively. (D) Presence of 5 mM ATP and 1 mM EDTA, Western blot analysis in the region of the δ subunit. All conditions are the same as in (C) above. (E) Presence of 5 mM MgATP, SDS−PAGE gels, Lane 1, control F_1; lane 2, control F_1 incubated at 37 °C for 8 h without trypsin; lanes 3−8, F_1 treated with trypsin for 0.5, 1, 2, 4, 6, and 8 h, respectively. (F) Presence of 5 mM MgATP, Western blot analysis in the region of the δ subunit. All conditions are the same as in (F). (G) Presence of 5 mM AMP·PNP, 5 mM ADP, and 5 mM $MgCl_2$. Lane 1, control F_1; lane 2, control F_1 incubated at 37 °C for 8 h without trypsin; lanes 3−8, F_1 treated with trypsin for 0.5, 1, 2, 4, 6, and 8, respectively. (H) Western blot analysis in the region of the δ subunit. All conditions are the same as in (G) above.

for the other subunits to dissociate from F_1 together with the δ subunit and appear in the filtrate. In other experiments not presented here, attempts to detect ATPase activity in the $\alpha_3\beta_3\gamma\epsilon$ retentate obtained at 0.0125% SDS failed, even after removal of the SDS.

These experiments, when taken together with those described above, indicate that the δ subunit is not deeply integrated into the central core of rat liver F_1 but rather is near the bottom of the molecule (Figure 9C). These experiments suggest also that the δ subunit may be required for F_1 stability.

DISCUSSION

The novel set of studies reported here provide important insights into the properties, location within F_1, and possible functions of the δ subunit of a higher eukaryotic ATP synthase. The model presented in Figure 9C is an attempt to rationalize the findings reported here. In the model the δ subunit is envisioned to reside at the bottom of the F_1 molecule, consistent with its selective removal by low concentrations of SDS (Figure 9A). Here, it is tightly bound and shielded in part by α, β, and γ subunits, consistent with studies using trypsin (Figure 7). Its C-terminal α-helical half, which is inaccessible to a C-terminal δ subunit antibody (Figure 5D) and to carboxypeptidase Y (Figure 6A), is depicted as "buried" near the α/β junction and its N-terminal β sheet/strand half is depicted as being enclosed by the γ subunit projecting out of the bottom of the molecule. The N-terminus of the δ subunit is shown to be exposed, consistent with its cross-reactivity with a monoclonal N-

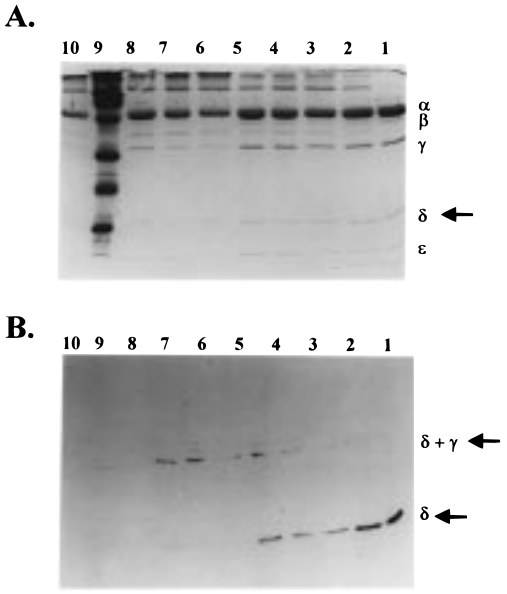

FIGURE 8: Formation of cross-links between the δ and γ subunits of rat liver F_1 in the presence of disuccinimidyl glutarate (DSG). Cross-linking studies with DSG were carried out using 60 μg of rat liver F_1 exactly as described under Methods and the resultant samples subjected to both SDS−PAGE and Western blot analysis using anti-δ antisera. A. SDS−PAGE gels of cross-linked products. Lane 1, control F_1 (no DSG); lane 2, F_1+ 0.0025 mM DSG, 30 min; lane 3, F_1 + 0.0125 mM DSG, 30 min; lane 4, F_1 + 0.0125 mM DSG, 15 min; lane 5, 0.0125 mM DSG, 5 min; lane 6, F_1 + 0.25 mM DSG, 30 min; lane 7, F_1 + 0.25 mM DSG, 15 min; lane 8, F_1 + 0.25 mM DSG, 5 min; lane 9, molecular weight markers; lane 10, F_1 + 0.31 mM DSG, 30 min. (The arrow designates the position of the δ subunit.) (B) Western blot analysis. After transferring an identical set of gels (unstained) to those in (A) to PVDF membranes, Western blot analysis was carried out using a δ antibody (JH-142) exactly as described under Methods. (The lower arrow designates the position of the δ subunit, while the top arrow designates the position of the δ + γ cross-link.)

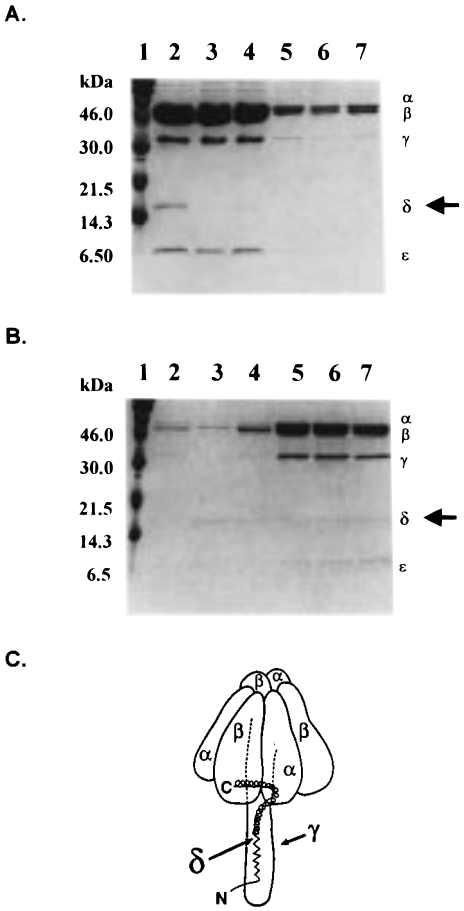

FIGURE 9: Selective removal of the δ subunit from rat liver F_1 using SDS. F_1 (50 μg) was prior treated with SDS exactly as described under Methods, placed in a tube containing a Microcon 100 filter, and subjected to centrifugation, also exactly as described under Methods. The flow-through fraction and the fraction retained on the membrane (after washing) were then analyzed by SDS−PAGE. (A) Fractions retained on the filter. Lane 1, molecular weight markers (unfiltered); lane 2, F_1, no prior treatment with SDS; lanes 3−7, F_1 pretreated with 0.0125, 0.025, 0.050, 0.10, and 0.20% SDS. (B) Flow-through fractions. Lanes are identical to in (A). In both (A) and (B) the arrow indicates the position of the δ subunit. (C) Model depicting the predicted location of the δ subunit near the bottom of the F_1 molecule. The secondary structure shown is that predicted and not that determined experimentally. The experimental values indicate that about 48% of the secondary structure is β-sheet/strand and about 19% is α-helix.

terminal δ subunit antibody (Figure 5F). As the catalytic cycle proceeds from the nucleotide free state, to the ATP bound state, and finally to the ATP hydrolytic (catalytic) state, different interactions of the δ and γ subunits are suggested to occur to account for the experimental data also obtained with trypsin (Figure 7).

It is both important and interesting to compare results obtained here on a higher eukaryotic F_1-δ subunit with those obtained previously on chloroplast and *E. coli* F_1-ϵ subunits, frequently considered as equivalent to higher eukaryotic δ subunits. Certainly, there are some striking similarities despite the fact that the rat liver δ subunit exhibits only about 25% identity in its amino acid sequence to the chloroplast and *E. coli* ϵ subunits. First, similar to the *E. coli* ϵ subunit, where it has been shown by NMR studies (*19*) that the N-terminal and C-terminal halves exhibit respectively β-sheet and α-helical secondary structure, the same "two domain" structural arrangement is predicted for the rat liver δ subunit (Figure 3A). Second, as is the case for the chloroplast and *E. coli* ϵ subunits (*18*, *51*, *52*), the rat liver δ subunit resides very near the γ subunit (Figure 8) and is predicted to also reside near the bottom of the F_1 molecule (Figure 9C). Third, the rat liver δ subunit, as the *E. coli* ϵ subunits (*12*−*14*), interacts with the β or catalytic subunit of F_1 (*50*). Finally, addition of nucleotides to *E. coli* F_1 affects the position/accessibility of the ϵ subunit (*14*, *15*), while addition of nucleotides to rat liver F_1 affects the position/accessibility of the δ subunit (Figure 7).

Despite these similarities, there are some notable differences. First, in contrast to the chloroplast and *E. coli* F_1 preparation where ethanol (*28*) and LDAO (*27*), respectively, induce removal of the ϵ subunit, these mild agents are unable to dissociate the δ subunit from rat liver F_1 (Figure 4). Rather, low concentrations of SDS are required to selectively induce removal of the rat liver δ subunit (Figure 9), consistent with a tighter interaction with the F_1 complex. Second, the ϵ subunit of *E. coli* F_1 is readily accessible to trypsin digestion (*53*), whereas the δ subunit of rat liver F_1 is much more resistant and occurs only after prior cleavage of the larger F_1 subunits (Figure 7A). Third, the ϵ subunits of both chloroplast and *E. coli* F_1 preparations are known to be ATPase inhibitors (*27*−*29*), whereas in rat liver F_1 a distinct protein called IF_1 binds F_1 and inhibits ATPase activity (*30*, *32*). Finally, after selective removal of the ϵ

subunit from chloroplast and *E. coli* F_1 preparations, the F_1-(-ϵ) preparations are catalytically active (*27*, *28*), whereas after selective removal of the rat liver F_1-δ subunit the resultant F_1(-δ) complex is inactive. (Although this inactivity may result because the δ subunit is essential for stabilizing the $\alpha_3\beta_3\gamma\epsilon$ complex, we cannot exclude the possibility that the low concentrations of SDS used inactivate the catalytic capacity of this complex while leaving it in an oligomeric state).

The above similarities and differences between the rat liver F_1-δ subunit and the F_1-ϵ subunits of chloroplast and *E. coli* may indicate that these structurally analogous subunit types play both similar and different roles in ATP synthase function. A common role is most likely in helping convey the energy conserved within an electrochemical proton gradient from the F_0 unit (proton channel) to the ATP synthesis site on F_1-β subunits. This view is supported by the findings that both subunit types appear to be closely associated with the functionally essential γ subunit, which is centrally located within the F_1 molecule (*33*) and rotates during catalysis (*24*). In fact, an interesting question is whether the δ subunit of higher eukaryotes and the ϵ subunits of chloroplast and *E. coli* rotate together with the γ subunit. (Although the "rotational" studies performed by Noji et al. (*24*) were carried out in the absence of the ϵ subunit, they do not exclude the possibility that this subunit rotates in bacterial F_1s and the δ subunit rotates in eukaryotic F_1s.) Different roles of the two subunit types may be related to the regulation and stabilization of F_1. Thus, in contrast to higher eukaryotic F_1 units where the distinct IF_1 peptide is an ATPase inhibitor (*30*, *31*), in chloroplast and *E. coli* the ϵ subunit is an inhibitor of F_1 ATPase activity (*27*, *28*). Conversely, in contrast to F_1 units from chloroplast and *E. coli* where the removal of the ϵ subunit enhances the ATPase function (*27*, *28*), removal of the δ subunit from rat liver F_1 results in an inactive complex. This may be related to a role of the δ subunit in stabilizing rat liver F_1, as studies with yeast containing δ subunit null mutations show that $\alpha\beta$ complexes are not formed (*53*, *54*). Thus, in yeast through higher eukaryotes one of the roles of the δ subunit may be as a "keystone" helping bridge α/β and γ subunits near the bottom central region of F_1.

Finally, two other studies bear mention in relation to work reported here. First, in an earlier brief report (*32*), the preparation of three monoclonal antibodies to the bovine heart F_1-δ subunit was described. Of these, two reacted with the N-terminus (Ala 1 through Met 16) of the isolated δ subunit and also showed reactivity toward the δ subunit in the intact bovine heart F_1. The third antibody reacted with Ser 17 through Glu 68 of the isolated δ subunit, but reactivity with the δ subunit within the intact bovine F_1 was hindered. These results are entirely consistent with the more detailed study reported here on rat liver F_1, which show that, with exception of the N-terminal region, the δ subunit is not readily accessible. In a second related study carried out in our laboratory after work reported here was completed, we compared the N-terminal sequence of the δ subunit in our purified rat liver F_1 preparation (*41*) with that of rat liver F_0F_1 prepared exactly as we previously described (*55*). The rat liver F_1-δ sequence determined by the Edman method (*37*, *38*) and as described earlier in this report is ASPAPA- - -, whereas that obtained more recently for the δ subunit within F_0F_1 is AQAAASPA- -. Comparison of these two sequences immediately shows that the first four amino acids present in F_0F_1 are missing in F_1, indicating that during our F_1 preparation these residues have been proteolytically cleaved. These results suggest that in F_0F_1 the first four N-terminal amino acids, AQAA, are protected by F_0, implicating an interaction of the δ subunit N-terminus with one of the F_0 subunits. These findings add additional support for the model presented in Figure 9C, which places the N-terminus of the δ subunit of rat liver F_1 at the very bottom of the F_1 molecule. Future studies will be directed at better understanding the chemical nature of this interaction.

ACKNOWLEDGMENT

We are grateful to Dr. Joel Lunardi of the Université Joseph Fourier for generously providing the δ subunit N-terminal monoclonal antibody, to Joanne Hullihen for technical support, to Dr. Saroj Mathupala for many helpful discussions and comments, to Dr. W. S. Liu and Jodie Franklin for N-terminal sequencing analyses, and to Jackie Seidl for help in processing the manuscript for publication.

REFERENCES

1. Penefsky, H. S., and Cross, R. L. (1991) *Adv. Enzymol. Relat. Areas Mol. Biol. 64*, 173-214.
2. Futai, M., Iwamoto, A., Omote, H., Orita, Y., Shin, K., Nakamoto, R. K., and Maeda, M. (1992) *J. Exp. Biol.* 443−449.
3. Pedersen, P. L., and Amzel, L. M. (1993) *J. Biol. Chem. 268*, 9937−9940.
4. Capaldi, R. A., Aggeler, R., Turina, P., and Wilkens, S. (1994) *Trends in Biochem. Sci. 12*, 186−189.
5. Fillingame, R. H. (1997) *J. Exp. Biol. 200*, 217−224.
6. Weber, J., and Senior, A. E. (1997) *Biochim. Biophys. Acta 1319*, 19−58.
7. Walker, J. E. Lutter, R., Dupuis, A., and Runswick, M. J. (1991) *Biochemistry 30*, 5369−5378.
8. Belogrudov, G. I., Tomich. J. M., and Hatefi, Y. (1996) *J. Biol. Chem. 271*, 20340−20345.
9. Soper, J. W., Decker, G. L., and Pedersen, P. L. (1979) *J. Biol. Chem. 254*, 11170−11176.
10. Gogol, E. P., Lucken, U., and Capaldi, R. A. (1987) *FEBS Lett. 219*, 274−278.
11. Kuki, M., Noumi, T., Maeda, M., Amenura, A., and Futai, M. (1988) *J. Biol. Chem. 263*, 17437−17442.
12. Mendel-Hartig, J., and Capaldi, R. A. (1991) *Biochemistry 30*, 1278−1284.
13. Mendel-Hartig, J., and Capaldi, R. A. (1991) *Biochemistry 30*, 10987−10991.
14. Aggeler, R., Haughton, M. A., and Capaldi, R. A. (1995) *J. Biol. Chem. 270*, 9185−9191.
15. Aggeler, R., and Capaldi, R. A. (1996) *J. Biol. Chem. 271*, 13888−13891.
16. Zhang, Y., Oldenberg, M., and Fillingame, R. H. (1994) *J. Biol. Chem. 269*, 10221−10224.
17. Dunn, S. (1982) *J. Biol. Chem. 257*, 7354−7359.
18. Tang, C., and Capaldi, R. A. (1996) *J. Biol. Chem. 271*, 3018−3024.
19. Wilkens, S., Dahlquist, F., McIntosh, L. P., Donaldson, L. W., and Capaldi, R. A. (1995) *Nat. Struct. Biol. 2*, 961−967.
20. Tozer, R. G., and Dunn, S. D. (1987) *J. Biol. Chem. 262*, 10706−10711.
21. Gogol, E. P., Johnston, E., Aggeler, R., and Capaldi, R. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9585−9589.
22. Duncan, T. M., Bulygin, V., Zhou, Y., Hutcheon, M. L., and Cross, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10964−10968.
23. Sabbert, D., Engelbrecht, S., and Junge, W. (1996) *Nature*, 623−625.

24. Noji, H., Yoshida, R., Yoshida, M., and Kinosita, K. Jr. (1997) *Nature 386*, 299−302.
25. Kagawa, Y., and Hamamoto, T. (1997) *Biochem. Biophys. Res. Commun. 240*, 247−256.
26. Dunn, S. D. (1986) *Anal. Biochem. 150*, 35−42.
27. Lotscher, H. R., de Jong, C., and Capaldi, R. (1984) *Biochemistry 23*, 4140−4143.
28. Richter, M. L., Patrie, W. J., and McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7271−7373.
29. Dunn, S. D., Zadorozny, V. D., Tozer, R. G., and Orr, L. (1987) *Biochemistry 26*, 4488−4493.
30. Pullman, M. E., and Monroy, G. C. (1963) *J. Biol. Chem. 238*, 3762−3769.
31. Cintrón, N. M., and Pedersen, P. L. (1979) *J. Biol. Chem. 254*, 3439−3443.
32. Bianchet, M., Ysern, X., Hullihen, J., Pedersen, P. L., and Amzel, L. M. (1991) *J. Biol. Chem. 266*, 21197−21201.
33. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature 370*, 621−628.
34. Lunardi, J., Dupuis, A., Frobert, Y., Grassi, J., and Bignai, P. V. (1989) *FEBS Lett. 245*, 223−228.
35. Kraft, R., Tardiff, J., Krauter, K. S., and Leinwand, L. A. (1988) *Biotechniques 6*, 544−547.
36. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
37. Edman, P. (1950) *Acta Chem. Scand. 4*, 283−293.
38. Hunkapiller, M. W., and Hood, L. E. (1983) *Science 219*, 650−659.
39. Chang, C. T., Wu, C.-S. C., and Yang, J. T. (1978) *Anal. Biochem. 91*, 13−31.
40. Merrifield, R. B. (1968) *J. Am. Chem. Soc. 85*, 2149−2154.
41. Catterall, W. A., and Pedersen, P. L. (1971) *J. Biol. Chem. 246*, 4987−4994.
42. Pedersen, P. L., Hullihen, J., and Wehrle, J. P. (1981) *J. Biol. Chem. 256*, 1362−1369.
43. Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960) *J. Biol. Chem. 235*, 3322−3329.
44. Laemmli, U. K. (1970) *Nature 227*, 680−685.
45. Runswick, M. J., Medd, S. M., and Walker, J. E. (1990) *Biochem. J. 266*, 421−426.
46. Saraste, M., Gay, N. J., Eberle, A., Runswick, M. J., and Walker, J. E. (1981) *Nucleic Acids Res. 9*, 5287−5296.
47. Zurawski, G., Bottomley, W., and Whitfeld, P. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6260−6264.
48. Kanazawa, H., Kayano, T., Kiyasu, T., and Futai, M. (1982) *Biochem. Biophys. Res. Commun. 105*, 1257−1264.
49. Garnier, J., Osquthorpe, and D. J. Robson, B. (1978) *J. Mol. Biol. 120*, 97−120.
50. Pedersen, P. L., Hullihen, J., Bianchet, M., Amzel, L. M., and Lebowitz, M. S. (1995) *J. Biol. Chem. 270*, 1775−1784.
51. Shuman, J., Richter, M. L., and McCarty, R. E. (1985) *J. Biol. Chem. 260*, 11817−11823.
52. Shapiro, A. B., Gibson, G. D. Scheraga, H. A., and McCarty, R. E. (1991) *J. Biol. Chem. 266*, 17276−17285.
53. Gavilanes-Ruiz, M., Tommasino, M., and Capaldi, R. A. (1988) *Biochemistry 27*, 603−609.
54. Giraud, M.-F., and Velours, J. (1994) *Eur. J. Biochem. 222*, 851−859.
55. McEnery, M., Buhle, E. L., Jr., Aebi, U., and Pedersen, P. L. (1984) *J. Biol. Chem. 259*, 4642−4651.

BI9800698