Mitochondrial F₀F₁ ATP Synthase

STUDIES REPORTED HERE WERE UNDERTAKEN TO OBTAIN GREATER MOLECULAR INSIGHT INTO THE COMPLEX STRUCTURE OF ATP SYNTHASE (F₀F₁) AND ITS RELATIONSHIP TO THE ENZYME'S FUNCTION AND MOTOR-RELATED PROPERTIES. SIGNIFICANTLY, THESE STUDIES, WHICH EMPLOYED N-TERTIARY SEQUENCE, MASS SPECTRAL, PROTEOLYTIC, IMMUNOLOGICAL, AND FUNCTIONAL ANALYSES, LED TO THE FOLLOWING NOVEL FINDINGS. FIRST, AT THE TOP OF F₁ WITHIN F₀F₁, ALL SIX N-TERMINAL REGIONS DERIVED FROM α + β SUBUNIT ARE SHIELDED, INDICATING THAT ONE OR MORE F₀ SUBUNIT FORMS A "CAP." SECOND, AT THE BOTTOM OF F₁ WITHIN F₀F₁, THE N-TERMINAL REGION OF THE SINGLE δ SUBUNIT AND THE C-TERMINAL REGIONS OF ALL THREE α SUBUNIT ARE SHIELDED ALSO BY F₀. THIRD, AND IN CONTRAST, PART OF THE γ SUBUNIT LOCATED AT THE BOTTOM OF F₁ IS ALREADY SHIELDED IN F₁, INDICATING THAT THERE IS A PREFERENTIAL PROPENSITY FOR INTERACTION WITH OTHER F₁ SUBUNIT, MOST LIKELY δ AND ε. FOURTH, AND CONSISTENT WITH THE FIRST TWO CONCLUSIONS ABOVE THAT SPECIFIC REGIONS AT THE TOP AND BOTTOM OF F₁ ARE SHIELDED BY F₀, FURTHER PROTEOLYTIC SHAVING OF α AND β SUBUNIT AT THESE LOCATIONS ELIMINATES THE CAPACITY OF F₁ TO COUPLE A PROTON GRADIENT TO ATP SYNTHESIS. FINALLY, EVIDENCE WAS OBTAINED THAT THE F₀ SUBUNIT CALLED "F₀′," UNIQUE TO ANIMAL ATP SYNTHASES, IS INVOLVED IN SHIELDED F₁. THE SIGNIFICANCE OF THE STUDIES REPORTED HERE, IN RELATION TO CURRENT VIEWS ABOUT ATP SYNTHASE STRUCTURE AND FUNCTION IN ANIMAL MITOCHONDRIA, IS DISCUSSED.

ATP synthases represent one of nature's most unique enzyme classes (reviewed in Refs. 1–3). Structurally, these enzymes are observed by electron microscopy (4–12) to consists of four distinct features, a headpiece, a basepiece, a central stalk or "stem region" connecting the headpiece and basepiece, and a "second stalk" extending from the basepiece to the top of the headpiece (4–10). Animal ATP synthases contain, in addition, what appears to be a fifth structural feature, a ring-like disc or "collar" surrounding the central stalk (11). Biochemically, however, ATP synthases separate most readily into only two units. One is a water-soluble component, F₁, containing five subunit types in the ratio αβγδε, whereas the other is a detergent-soluble component, F₀, containing three subunit types (α, β, ε) in bacteria and at least 10 more in animal systems (1–3). A regulatory protein, IF₁, is found also in isolated mitochondrial ATP synthases. A variety of studies involving biochemical (13–25), electron microscopic (4–12), and x-ray crystallographic (26–30) approaches, have shown that the headpiece and central stalk are derived primarily from F₁ subunits and the basepiece and second stalk are derived primarily from F₀ subunits. In contrast, the subunit source of the ring-like disc or "collar" surrounding the central stalk in animal ATP synthases (11) remains unknown.

Nature's rationale for providing ATP synthases with this unique molecular architecture has become apparent only in recent studies where evidence that these fascinating enzymes contain two motors has been mounting (31–33). One of the proposed motors is F₁, which is driven by ATP binding and/or hydrolysis (31), whereas the other is contained within F₀ and driven by a proton gradient (33). During ATP synthesis, the proposed motor within F₀, composed of 10–12 subunit c molecules (30, 34) and a single subunit a, is believed to drive the F₁ motor in reverse (35) via a central rotor, the F₁-γ subunit. This subunit extends from a ring of subunit c molecules in the F₀ basepiece (19), through the central stalk region, and finally through the center of F₁ where it interacts differently with each of the three αβ pairs (26–29). During one 360° rotation of the γ subunit, the binding of ADP and inorganic phosphate, the synthesis of ATP, and the release of this ATP are believed to occur on each αβ pair as proposed by the "binding change mechanism" (2, 36). During this process, the ε subunit in bacteria, or its δ subunit equivalent in animals, is also believed to rotate (32), presumably at the bottom of F₁ within the central stalk region.

Although much has been learned about the structure and function of ATP synthases as summarized above, a three-dimensional structure of the complete enzyme (F₀F₁) from any source. In fact, of the 16 different subunit types within the mitochondrial ATP synthase of animal cells, only two and a half of these (α, β, and about half of γ) have been solved at atomic resolution (26, 28). Consequently, there are still many important questions that remain unanswered about structure/function relationships in ATP synthases, and how, within these remarkably complex machines, energy coupling between the proposed proton gradient-driven motor within F₀ is coupled to the reversal of the ATP-driven F₁ motor. Specifically, one important question relates to the identity of exterior subunit regions at the top and bottom of F₁ that are shielded by F₀ in the complete ATP synthase, because these regions may include F₀/F₁ contact sites essential for energy coupling during ATP synthesis. Although information about these regions, particularly at or near the top of F₁, is gradually

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being generated for the *Escherichia coli* and chloroplast enzymes (12, 23), little information is available for animal ATP synthases.

In studies reported below, we used a variety of approaches to identify exterior subunit regions at the top and bottom of rat liver F₁ that are shielded by Fₐ. We then assessed the requirement of these regions for coupling a proton gradient to ATP synthesis. In addition, we obtained evidence for the involvement of an F₀ subunit unique to animal ATP synthases.

**EXPERIMENTAL PROCEDURES**

**Materials**

Rats (Harlan Sprague-Dawley®), white males were obtained from Charles River Breeding Laboratories. ATP, MgCl₂, EDTA, DTT, tricine, Sephadex G-25 (coarse), soybean trypsin inhibitor, oligomycin, and DCCD were from Sigma. SDS, acrylamide, and bisacrylamide were from Bio-Rad, PVDF membranes from Millipore, and Western blot reagents from Amersham Pharmacia Biotech. Modified trypsin (sequencing grade), pyrogallolamine aminopeptidase, hexokinase, and glucose-6-phosphate dehydrogenase were from Roche Molecular Biochemicals. The detergent CHAPS was obtained from Anatrace, whereas succrose, urea, and potassium phosphate were from J.T. Baker. Coomassie Blue dye binding reagent for protein determination was from Pierce, AEBSF was from Calbiochem, and 3,5-dimethoxy-4-hydroxy-cinnamic acid and α-cyano-4-hydroxy-cinnamic acid were from Aldrich. Centriprep filtration units for protein concentration were from Amicon. Venturicidin was from BDH Chemicals. The antibodies to Fₐ components were generous gifts from Ds. Y. Hatefi and Akemi Matsuno Yagi. All other products were of the highest purity or grade commercially available.

**Methods**

**Purification of Mitochondrial ATP Synthase (F₁,Fₐ).**—This complex was purified from rat liver mitochondria by a modification of a previously described method developed in this laboratory (8). A washed subconfluent mitochondrial fraction, referred to previously as 3X membranes (6), was stored at −15 mg/ml and −20 °C in TA buffer (50 mM Tricine, 1 mM ATP, 25 mM EDTA, 0.5 mM DTT, and 5% ethylene glycol, pH 7.9), thawed, and solubilized at 4 mg/ml in 0.6% CHAPS for 1 h on ice with occasional stirring. After centrifugation for 1 h at 48,000 rpm at 4 °C in a 70.1-Ti rotor in a Beckman Optima LE 80K ultracentrifuge to remove unsolubilized material, the supernatant was concentrated using Amicon's Centriprep filtration unit (molecular mass cutoff 10,000). The concentrate was diluted with TA buffer to give a CHAPS concentration of 0.1% and frozen in dry ice and ethanol, and then stored at −20 °C for 3 h. The sample (∼20 ml) was then thawed, and 5-mol aliquots (2.0–2.5 ml) were placed on 20 ml of 25% sucrose in TA buffer without CHAPS and centrifuged 12 h and 45 min at 4 °C in the same conditions. The pellets above buffer were then transferred to a TA buffer containing 0.1% CHAPS, 5 mM MgCl₂, 2 mM ATP, 200 mM sucrose, 20 IU hexokinase, 0.25 IU glucose-6-phosphate dehydrogenase, and 0.10–0.20 μg of inner mitochondrial membrane vesicles. The reaction was started by the addition of 1 ml ATP.

**SDS-PAGE.**—This was carried out as indicated either by the method of Laemmli (40) or the more sensitive method of Schägger and von Jagow (41) for separating proteins in the molecular mass range of from 1 to 100 kDa.

**Western Analysis.**—After conducting SDS-PAGE, the proteins on the gel were transferred electrophoretically at 4 °C in 10 mM CAPS, 10% methanol transfer buffer, pH 11, onto a PVDF membrane (1 h at 100 V and 0.2 amp). The membrane was then blocked for 1 h with 2% bovine albumin plus 5% non-fat dry milk in phosphate-buffered saline T buffer (bath at 4 °C). The membrane was then incubated with the primary antibody (1 h at 4 °C) and then washed with T buffer (bath at 4 °C). The membrane was then incubated with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG). The immunoreactive bands were detected by an enhanced chemiluminescence (ECL) system.

**Mass Spectral Analysis.**—Mass spectra were acquired on samples of 10–20 pmol in a PerSeptive Biosystems Voyager MALDI-TOF-DE mass spectrometer using a nitrogen laser (wavelength 337 nm). The matrix was either 3,5-dimethoxy-4-hydroxy-cinnamic acid or α-cyano-4-hydroxy-cinnamic acid. Positive ion mass spectra were analyzed using PerSeptive GRAMS software version 3.01c.

**Protein Determination.**—Protein determination was done by the method of Lowry et al. (44), biuret (45), and Coomassie dye (Pierce). In all cases bovine albumin was used as the standard.

**RESULTS**

**Objectives and Systems Employed.**—The first objective of this study was to identify exterior regions on F₁ that are shielded by Fₐ within a fully active, complete ATP synthase (F₁,Fₐ) preparation from an animal mitochondrial source. The regions on F₁ of the transmembrane unit (Fig. 1) were the top where the N-terminal regions of α and β subunits reside (26–28) and its bottom where the C-terminal regions of these same subunits (26–28) reside together with part of the γ subunit (26, 28), the δ subunit (25, 30), and likely also the ε subunit. The second objective was to assess the importance of these regions for coupling an electrochemical proton gradient to ATP synthesis. This information is generally lacking for animal ATP synthases, which are much more complex in their Fₐ subunit composition than related enzymes from *E. coli* and chloroplasts. The third objective was...
to identify at least one $F_0$ subunit unique to the animal enzyme involved in shielding $F_1$, because such a subunit would represent a candidate for part of the stator or second stalk believed to stabilize the $F_1$ motor. To complete these objectives, it was essential to have in hand, in addition to our extensively studied rat liver $F_1$ preparation (25, 28, 37–39), an intact, highly purified preparation of the complete ATP synthase from the same source. This was accomplished by introducing several modifications (see “Methods”) into the CHAPS-based procedure previously developed in this laboratory (6). The resultant prep-
**Evidence That the N-terminal Regions of the α and β Subunits Located at the Top of F₁ Are Shielded by One or More F₀ Subunits in the Complete ATP Synthase**—Here, studies focused on the N-terminal regions of the three α and three β subunits as they are known from the atomic resolution structures of bovine heart and rat liver F₁ (Fig. 1A) to project from the top of the enzyme (26, 28). To determine whether one or more of the six N-terminal regions derived from these subunits are shielded by F₀, we compared the extent to which F₁, both alone and as part of the complete ATP synthase complex (F₀F₁), is shaved by endogenous proteases during purification. This necessitated carrying out N-terminal sequence analysis on both the α and β subunits of preparations of rat liver F₁ and F₀F₁ purified as described under “Methods.” Therefore, following purification, these subunits were transferred from SDS-PAGE gels onto PVDF membranes by electroblotting, excised, and subjected to sequence analyses, also as described under “Methods.”

In these experiments, the N-terminal regions of α subunits derived from the F₀F₁ preparation could be sequenced only following prior treatment with the enzyme pyroglutamate aminopeptidase (48), which removes from the blocked N termini pyrrolidone carboxylic acid (Fig. 2A, inset), a cyclic form of glutamine. The results of these experiments revealed that the N-terminal regions of all α and β subunits remain intact in F₀F₁ during its purification from mitochondria, because the amino acid sequences obtained (Fig. 2, A and B) correspond to those predicted for the mature sequences of these subunits. In contrast, the same subunits are shaved during the purification of F₁ (Fig. 2, C and D). Thus, two amino acids, Q and K, are shaved from α subunits, whereas the 6-amino acid peptide, AAAQSSA, is shaved from β subunits (Fig. 2E). The degree of shaving remained the same in a number of F₁ preparations examined. In all cases the N-terminal sequence data obtained was homogeneous, thus ruling out the possibility of differences among the N-terminal regions of each of the three α subunits and each of the three β subunits. These results indicate that, when within the complete ATP synthase, all six N-terminal regions of α + β subunits located at the top of F₁ are shielded during purification from specific proteases endogenous to the mitochondria. Moreover, they strongly implicate one or more F₀ subunits as comprising this shield, which appears to “cap” the top of F₁ rather than being confined to a limited region. Consistent with these conclusions, in experiments not reported here, treatment of purified F₀F₁ with trypsin for 1.5 h was unable to cleave the N-terminal regions of either the F₁ α or β subunits despite the presence of potential cleavage sites (R and/or K) in both cases.

**Evidence That, of the Three Remaining Subunits (γ, δ, and ε), Only the N-terminal Region of δ, Located at the Bottom of F₁, Is Shielded by One or More F₀ Subunits in the Complete ATP Synthase**—A comparison of amino acid sequencing data presented in Figs. 3A and 3B, and summarized in 3G, shows that the N-terminal region of the γ subunit has not been shaved by proteases during isolation of F₁. Rather, its N-terminal sequence is identical to that found in purified F₀F₁, and to the known sequence for this subunit. This is an expected result, which serves as a control. Thus, it is known from the atomic resolution structures of bovine heart and rat liver F₁ preparations (26, 28) that the N-terminal region of the γ subunit is tucked deep inside the central cavity of the F₁ headpiece where it is well shielded. In contrast, sequencing data for the δ subunit presented in Figs. 3C and 3D, and summarized in 3G, show that in F₁ purified alone a 4-amino acid peptide, AQAA, is shaved from its N-terminal region. However, when the δ subunit is purified as part of the complete F₀F₁ complex, the AQAA peptide is retained. Finally, a comparison of N-terminal sequencing data presented in Figs. 3E and 3F, and summarized also in Fig. 3G, show that the ε subunit, just as the γ subunit, has not been shaved during isolation, indicating that it may be shielded also within F₁. To date, little is known about the precise location and role of the ε subunit in mitochondrial ATP synthases, although it has been reported to subfractionate with a βδ complex (49) and to bind to the δ subunit (50).

The studies described here, taken together with the above studies, indicate that, within the complete liver mitochondrial ATP synthase, regions of F₀ located at both the top and bottom of F₁ shield the N-terminal regions of three of its subunit types (α, β, and δ), whereas the remaining two subunit types (γ and ε) are shielded or tightly folded within F₁.

**Limited Treatment of Isolated F₁ with Trypsin Results in Further Shaving of Its α and β Subunits While Leaving the Smaller Subunits γ, δ, and ε Unaltered**—Results presented in Fig. 4 (A and B) show that treatment of isolated F₁ with trypsin for as long as 90 min has no effect on the staining intensities or mobilities of the γ, δ, and ε subunits of F₁ in SDS-PAGE gels but does noticeably affect the mobility of the α subunit. Two different gel systems were used in these experiments. One
Because the decrease in mass of the subunit appeared by trypsin treatment of the complete ATP synthase (F₀F₁), in contrast to treatment of F₁, had no detectable effect on the electrophoretic mobility of the α subunit in SDS-PAGE (Fig. 5C), indicating that F₀ shields the C-terminal regions of all three of these subunits near the bottom of the F₁ headpiece.

Trypsin-treated F₁, Although Active as an ATPase and Capable of Rebinding to F₀ within F₁-depleted Inner Mitochon-

Fig. 3. A and B, N-terminal sequence analyses obtained for the γ subunit within purified preparations of F₀F₁ (A) and F₁ (B). Preparation of samples for N-terminal sequence analyses and the sequencing method used are described under “Methods.” C and D, N-terminal sequences obtained for the δ subunit within purified preparations of F₀F₁ (C) and F₁ (D). Preparation of samples for N-terminal sequence analyses and the sequencing method used are described under “Methods.” E and F, N-terminal sequences obtained for the ε subunit within purified preparations of F₀F₁ (E) and F₁ (F). Preparation of samples for N-terminal sequence analyses and the sequencing method used are described under “Methods.” G, summary of data not presented here, the N-terminal sequences obtained for the γ, δ, and ε subunits of F₀F₁ and F₁ but not F₂F₃.

contained 7.5% acrylamide (Fig. 4A), which separates best the α, β, and γ subunits but not the δ and ε subunits, which migrate with the dye front. The second contained 15% acrylamide (Fig. 4B), which separates the γ, δ, and ε subunits better than the α and β subunits.

When all five F₁ subunits from the SDS-PAGE gels were subjected to N-terminal sequence analyses, it was found that a 13-amino acid peptide (GTGTAEMSSILEER) had been cleaved from the N-terminal region of the α subunit (Fig. 4C) and a 3-amino acid peptide (APK) from the N-terminal region of the β subunit (Fig. 4D). In data not presented here, the N-terminal regions of the γ, δ, and ε subunits were found to be unaltered. Because the decrease in mass of the α subunit appeared by inspection of SDS-PAGE gels (Fig. 4, A and B) to be greater than the mass of the 13-amino acid peptide cleaved from its N terminus, this suggested that trypsin may have cleaved also at the C terminus. For this reason we subjected both isolated F₁ and trypsin-treated F₁ to MALDI-TOF-DE mass spectral analysis as described under “Methods.” Consistent with SDS-PAGE and N-terminal sequence analyses, these studies confirmed that the mass of the γ, δ, and ε subunits were unaltered and showed that the mass of each β subunit had decreased by only 341 Da (Fig. 5A), as expected from the loss of the APK peptide. In contrast, however, the mass of each α subunit decreased by 4425 Da, of which only 1406 Da could be accounted for by the 13-amino acid peptide cleaved from the N-terminal region (Fig. 4C). Significantly, the remaining decrease in mass, which must come from the C-terminal region, corresponds most closely to a 26-amino acid α-helix resulting from trypsin cleavage between amino acid Arg-484 and Ser-485 (Fig. 5B).

These results, while demonstrating that limited trypsin treatment of isolated F₁ causes further shaving of the exterior N-terminal regions of both the α and β subunits located at the top of the molecule, and the exterior C-terminal helix of the α subunit located at its bottom, also show that all three of the smaller subunits (γ, δ, and ε) are resistant to limited trypsin treatment. Significantly, in other studies it was shown that trypsin treatment of the complete ATP synthase (F₀F₁), in contrast to treatment of F₁, had no detectable effect on the electrophoretic mobility of the α subunit in SDS-PAGE (Fig. 5C), indicating that F₀ shields the C-terminal regions of all three of these subunits near the bottom of the F₁ headpiece.
Fig. 5. A, summary of molecular masses obtained for F1 subunits before and after limited treatment with trypsin. F1 was subjected to limited treatment with trypsin for 1 h exactly as described under "Methods" and then analyzed in a MALDI-TOF-DE mass spectrometer, also as described under "Methods." The masses obtained from two different experiments varied less than 1%.

B, predicted trypsin cleavage site within the C-terminal region of the α subunit (RS84) at the bottom of F1.

C, lack of effect of trypsin on the mobility of the α and β subunits within purified F1. F1, F0F1, was treated for up to 1.5 h with trypsin as described under "Methods" and then subjected to SDS-PAGE, also as described under "Methods."

Results presented in Fig. 6A show that regions under which trypsin further shaves the N-terminal regions of both α and β subunits at the top of rat liver F1, and the C-terminal region of the α subunits at its bottom (Figs. 4 and 5), are without effect on the capacity of the enzyme to catalyze ATP hydrolysis. Moreover, similar to isolated F1, trypsin-treated F1 is able to restore to F1-depleted inner membrane vesicles (IMVs) the capacity to catalyze high rates of ATP hydrolysis and ATP synthetic activities. Preparation of F1-depleted inner membrane vesicles by treating inner membrane vesicles with 3.2 M urea, and reconstitution of F1 (300 μg) with trypsin-treated F1 (300 μg) with the depleted vesicles (0.5 mg), was carried out exactly as described previously (39). ATPase and ATP synthetic activities were assayed as described under "Methods." Where indicated, oligomycin (2.5 μg/ml), DCCD (5 μg/ml), or 2,4-dinitrophenol (100 μM) was added. Standard deviations are based on three different experiments. (In experiments not presented here, DCCD (5 μg/ml) inhibited by >90% the rates of ATP hydrolysis and ATP synthase in all cases.)

Evidence for an Involvement of the Unique F0 Subunit Called F6 in Shielding F1—Having acquired information that regions on rat liver F1, at its top and bottom, are shielded by F0, and that one or both of these locations on F1 may be necessary for coupling a proton gradient to ATP synthesis, our attention turned to the involvement of F0. Here the major goal was to identify one or more F0 subunits unique to the animal system involved in shielding F1 within the complete F0/F1 ATP synthase. The F0 subunits named “F6,” “OSCP,” and “d” were considered to be likely candidates, because these subunits are not integral membrane proteins (18, 51, 52). The strategy employed took advantage of our knowledge that the N-terminal regions of each α subunit shielded by F0 at the top of F1 has its N terminus blocked by pyrrolidone carboxylic acid. Therefore, it was rationalized that, by monitoring the ability of a deblocking enzyme to gain entrance to the N terminus of the blocked α subunit in F0/F1 after the addition of a mild dissociating agent, while monitoring the release of any F0 subunits, we might be able to identify one or more F0 subunits involved in shielding F1.

Based on the above strategy and considerable preliminary work, it was found that the deblocking enzyme (pyroglutamate aminopeptidase) gains access to the N terminus of the α subunit after treatment of F0/F1 with 0.6% CHAPS for 1 h (12 h at 4°C and then 2 h at 25°C). Thus, the N-terminal region of the
α subunit following SDS-PAGE and electroblotting onto a PVDF membrane could now be sequenced (Fig. 7A), verifying that deblocking had occurred. Significantly, 1 M urea, when included under the above conditions, actually stabilized F₉F₁ and prevented the deblocking enzyme from gaining access to the N terminus of the α subunit (Fig. 7B). When both the supernatant and pellet following treatment of F₀F₁ with CHAPS, but without urea, was analyzed by immunoblotting with an antibody specific for F₉, all of this subunit was recovered in the supernatant (Fig. 7C, Condition 1). In contrast, when 1 M urea was present, F₉ was not detected in the supernatant (Fig. 7C, Condition 2) but remained with the pellet. Experiments conducted in an identical manner but using antibodies specific for OSCP, showed that this subunit is also shielded from proteolysis arising from endogenous proteases during purification. Clearly, as revealed by N-terminal sequence analyses (Figs. 1 and 2), exterior regions at both the top and bottom of rat liver F₁ within the complete ATP synthase are shielded from limited proteolysis arising from endogenous proteases during purification. Shielded regions include the N-terminal regions of all α and β subunits, which are known from the atomic resolution structure of rat liver F₁ to reside at its top (28), and the N-terminal region of the single δ subunit, which is inferred from biochemical studies to reside at its bottom (25). Treatment of the complete ATP synthase (F₇F₁) with trypsin is also without effect on the N-terminal regions of these same subunits, providing additional support that they are shielded by F₉. These findings, although identifying potential F₉/F₁ contact regions in the complete rat liver ATP synthase, also implicate F₁ as being “capped” at its top by one or more subunits derived from F₉ and shielded at its bottom.

Significantly, the exterior N-terminal regions of F₁, which are shielded from limited proteolysis during isolation of F₇F₁, i.e. the QK dipeptide of the α subunit (Fig. 2E), the AAQSSA hexapeptide of the β subunit (Fig. 2E), and the AQAA tetrapeptide of the δ subunit (Fig. 3G), are not required for F₁ to restore ATP synthesis to F₀-depleted inner membrane vesicles (Fig. 6B). However, if the endogenously shaved F₁ preparation is shaved further at both its top and bottom by exogenously added trypsin, the enzyme is unable to catalyze ATP synthesis when reconstituted with F₀-depleted inner membrane vesicles (Fig. 6B). This loss of physiological function occurs despite the fact that the more closely shaved F₁ fully retains both its catalytic ATPase activity (Fig. 6A) and its capacity to bind in a normal manner to F₀-depleted inner membrane vesicles (Fig. 6B). Specifically, trypsin removes from the α subunit’s N-terminal region an additional 13 amino acids, TGTAEISSILLER (Fig. 4C), and from its C-terminal region a 26-amino acid α-helix, SDGKISEQSDAKLKEIVTNFLAGFEP (Fig. 5B), while removing also the tripeptide APK from the N-terminal region of the β subunit (Fig. 4D). Taken together, these findings summarized in Fig. 8A indicate that one or more of these trypsin-cleaved exterior regions, which are also shielded by F₉ when within the complete ATP synthase (Figs. 2, 3, and 5), are critical for the capacity of F₁ to couple an electrochemical proton gradient to the synthesis of ATP.

Additional results presented here provide new information about the relationship of F₉ in the complete ATP synthase to the central stalk region of F₁, i.e. the region that extends from the bottom of the F₁ headpiece to the basepiece of F₉, a distance of about 50 Å (30). From SDS-PAGE, N-terminal sequencing, and mass spectral studies following treatment with trypsin (Figs. 4 and 5), it is clear that γ subunit is already well protected in the central stalk region within F₁. This shield is likely provided, at least in part, by the δ subunit of F₁, a view that is supported by previous work in this laboratory on rat liver F₁ (25) and from the low resolution structure of yeast F₇ in complex with a decamer of the subunit c of F₉ (30). The ε subunit of F₇, which binds to the δ subunit (50), may also comprise, in part, the shield that protects the γ subunit within F₁. Because
likely subunits of F0. Although another location cannot be completely ruled out. F1 headpiece, perhaps forming a collar; and (a) that make the N-terminally blocked residue (Q*) of the unique F0 subunit F6 in helping shield F1, most likely at its top, F0 may reside sufficiently near the central stalk region to form in the intact ATP synthase complex, one or more components of F0 may be composed, at least in part, by the F0 subunit F6, unique to animal ATP synthases. This model, derived from using a variety of approaches and methodologies, is consistent with current views about ATP synthase structure, which envision the presence of a stator connecting two motors, one within the F1 headpiece and another within the F0 basepiece (31–33, 35). In addition, this model and the experimental work on which it is based provide biochemical support for the recent electron microscopic study of bovine heart ATP synthase, showing a collar around the central stalk and a mass at the top of the F1 (11), and support for a similar study conducted on the E. coli ATP synthase in which a mass was also observed on the top of F1 (12). Taken together, these studies suggest that earlier models (53–55) depicting the stator or second stalk as interacting with only one αβ pair and failing to extend from the basepiece of F0 to the top of F1 may require revision.

Significantly, the extension of the stator to the top of F1 may have important functional consequences, because not one but all three αβ pairs containing the catalytic sites must be stabilized during ATP synthesis. Moreover, that part of the stator that resides at the top may be important also in facilitating formation of those critical contacts recently suggested to be important in the assembly of the β-barrel domains (56).

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FIG. 8. A summary of exterior regions of F1 subunits shielded by F0 that are not essential for mitochondrial ATP synthase, and those of which one or more is essential. Blue italicized letters designate those exterior amino acid residues that are not essential, while red letters inside dotted boxes designate those of which one or more is essential. B, model of rat liver mitochondrial ATP synthase incorporating conclusions derived from the novel findings reported here. Experimental data reported here are consistent with a model for animal ATP synthases in which (a) one or more F0 subunits completely shields the top of F1 forming a cap; (b) one or more F0 subunits also shields the bottom of the F1 headpiece, perhaps forming a collar; and (c) components of F1, most likely δ and ε, shield the γ subunit within the region of the central stalk at the bottom of F1. The data reported are consistent also with a role for the unique F0 subunit F6 in helping shield F1, most likely at its top, although another location cannot be completely ruled out.
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