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The effect of mild trypsin digestion of F_1 on energy coupling in the mitochondrial ATP synthase

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Abstract Mild trypsin digestion of isolated bovine-heart mitochondrial F_1-ATPase removed the first 15 residues from the N-terminus of subunit α under conditions in which other F_1 subunits were apparently untouched. When the trypsinized F_1 (TF_1) was reconstituted with the F_0 sector in the mitochondrial membrane (USMP), the ATP hydrolase activity acquired oligomycin sensitivity but ATP hydrolysis was decoupled from proton pumping. TF_1 added to USMP did not block the proton channel in F_0 as the native F_1 did. AMP-PNP inhibited proton conductivity in reconstituted F_1-USMP but this effect was lost in reconstituted TF_1-USMP. These results indicate that the N-terminus of the F_1 α subunit plays a critical role in the conformational communication between F_1 and F_0.

Key words: ATP synthase; F_1; Trypsin digestion; Energy coupling

1. Introduction

The F_0F_1 ATP synthase, embedded in the coupling membrane of bacteria, mitochondria and chloroplasts, converts the energy of $\Delta\mu H^+$ into ATP synthesis and vice versa. The bovine heart mitochondrial ATP synthase contains 16 subunits which form: the catalytic sector, F_1, the proton translocating, F_0 sector and the stalk, connecting F_1 and F_0 [1,2]. It is widely accepted that energy coupling between F_1 and F_0 occurs via long range conformational interactions (the binding-change model) [3–5]. Although some evidence has appeared which supports a rotatory model, the molecular mechanism of the coupling is far from being clarified [6].

The high-resolution X-ray crystallographic structure of bovine heart F_1 shows that both α and β subunits consist of an N-terminal β barrel domain, a nucleotide binding domain and a C-terminal α helix bundle [7]. The amino acid sequences 1–19 and 1–8 in the α and β subunits, respectively, are not visible in the crystallographic structure, they are apparently disordered and located at the top of F_1 [7].

Mild trypsinolysis of isolated mitochondrial F_1 (IF_1) has been shown to remove the first 15 amino acids from the N-terminus of α subunit and about 6–7 residues from the N-terminus of β subunit [8], but to leave other subunits untouched [8–11]. This proteolytic cleavage did not affect ATP hydrolase activity of F_1 or its binding to F_0 in the membrane [10–12], but impaired energy-linked functions when F_1 was reconstituted with F_0 in the membrane [10–13]. Since the N-terminus in the α subunit appears to be structurally separated from the F_0 and the stalk subunits [7], how the removal of this region can affect the coupling and interaction between F_1 and stalk subunits is intriguing.

The present work shows that mild trypsinolysis of purified bovine-heart mitochondrial F_1-ATP synthase, causing selective cleavage of the N-terminus of the α subunit, impairs the functional interaction of F_1 with the proton channel in F_0 and coupling of ATP hydrolysis to proton translocation. These observations indicate a critical role of the N-terminus of F_1 α subunit in the interaction and energy coupling between F_1 and F_0.

2. Material and methods

2.1. Materials

Oligomycin, G-200, valinomycin, DEAE A-50, and AMP-PNP were from Sigma. Trypsin, trypsin inhibitor, NADH, ATP, pyruvate kinase, lactate dehydrogenase were from Boehringer. SDS, goat anti-rabbit IgG labeled with peroxidase, color developing reagent were from Bio-Rad. Nitrocellulose membrane (NCM) (0.45 μm pore size) was from Schleier and Schull Ltd. PVDF membranes (immobilon transfer) (0.45 μm pore size) were from Millipore and the reagent for sequence analysis from Applied System. ACMA was from Molecular Probes.

2.2. Methods

Beef heart mitochondria, ESMP and USMP were prepared as described in [14]. F_1 was purified according to Beechy et al. [15] and passed through a G-200 column at pH 8.0 to remove IF_1 and contaminants. F_1 was stored in 250 mM sucrose, 50 mM Tris, 2 mM EDTA, 4 mM ATP, final pH 8.0 and 50% saturated $(NH_4)_2SO_4$ at 4°C. Before use, F_1 was precipitated and passed through two centrifugation columns preequilibrated in 250 mM sucrose, 10 mM Tris, pH 7.4 as described [16].

Mild trypsinolysis was performed as follows: F_1 (2 mg/ml) was suspended in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 2 mM EDTA and incubated at room temperature with 10 μg/ml trypsin for different times. At the end of the incubation, the trypsin inhibitor was added in 5-fold excess with trypsin. The control F_1 was incubated with trypsin and trypsin inhibitor added together under the same conditions. Reconstitution of F_1 and trypsin-digested F_1 (TF_1) with USMP was as described in [17].

Electrophoresis in 12 or 12–20% gradient SDS-PAGE was performed as in [18]. The electrophoresis resolved proteins were electrotransferred to NCM and immunodetected according to [19]. N-terminal amino acid sequence was performed as described in [20].

The α and β subunits were separated according to [21]. The precipitate containing α and γ subunits was dissolved in 8 M urea, 20 mM Tris, pH 7.2, chromotographed on a DEAE A-50 (1×5 cm) column to remove the contaminant β subunit.

The ATP hydrolase activity was measured with an ATP-regenerating system as in [22]. Passive proton conduction, followed as the aerobic release of the respiratory proton gradient, was measured as

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Abbreviations: TF_1, 5 min trypsin-digested F_1; USMP, F_1-depleted submitochondrial particles; F_1-USMP, USMP reconstituted with F_1; TF_1-USMP, USMP reconstituted with TF_1; IF_1, ATP synthase inhibitor protein; ACMA, 9-amino-6-chloro-2-methoxyacridine

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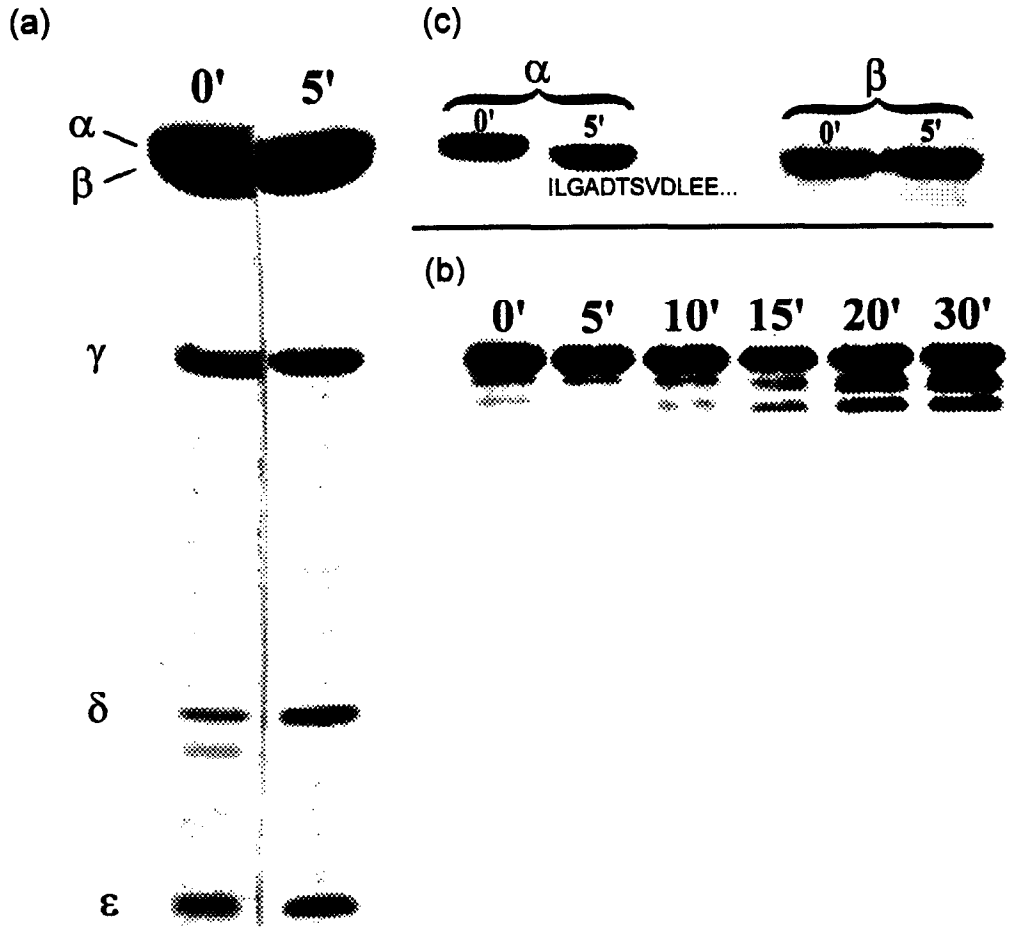

Fig. 1. SDS-PAGE and immunoblotting analysis of trypsin digestion of subunits of purified F_1-ATPase and of isolated α and β subunits from bovine heart mitochondria. F_1 was purified and exposed to trypsin digestion as described under Section 2. (a) 40 μg F_1 or F_1 digested by trypsin for 5 min were analysed by SDS-PAGE on a 12–20% gradient polyacrylamide as described in [18]. (b) 10 μg of F_1 and F_1 digested with trypsin for the times indicated, after SDS-PAGE, were electrotransferred on a nitrocellulose sheet and immunoblotted with anti-γ IgG as in [19]. (c) Subunits α and β were purified from control F_1 and F_1 digested for 5 min with trypsin, as described in Section 2 and subjected to SDS-PAGE. The product of trypsin digested subunit α was transferred on immobilon sheet and sequenced as described under Section 2.

in [23] and ATP-driven proton pumping was monitored by ACMA fluorescence quenching as in [24]. Proteins were determined by a modified Lowry method [25].

3. Results

Fig. 1a shows the SDS-PAGE pattern of F_1 subunits in the control and after 5 min incubation of F_1 with trypsin. Subunit α was completely digested to a product with the same M_r of the β subunit, which was apparently untouched. Prolonging the incubation with trypsin had no further effect on the α subunit, nor did it have any influence on the β subunit (not shown) (see also [9]). After 5 min incubation of F_1 with trypsin the electrophoretic pattern did not reveal any digestion of the other three subunits. After a longer incubation time with trypsin two bands appeared, corresponding to digestion products of the γ subunit (Fig. 1b). The α subunit of control F_1 and TF_1 was also separated from the β subunit, subjected to SDS-PAGE (Fig. 1c), transferred to immobilon and the N-terminus sequenced. The results (see inset in Fig. 1) showed that 5 min trypsin digestion removed the first 15 residues from the N-terminus of subunit α. The N-terminal segment of the native α subunit is: EKTGTAEVSSILEERILGADTSV-DLEE...[8].

Eadie-Hofstee plots of initial rates of ATP hydrolysis by F_1 and TF_1 showed, in both cases, negative cooperativity for

Table 1
K_m and V_{max} values for ATP hydrolysis by control F_1 and trypsin-digested F_1

	K_{m1} (μM)	V_{max1} (μmol P_i min^{-1} mg $protein^{-1}$)	K_{m2} (μM)	V_{max2} (μmol P_i min^{-1} mg $protein^{-1}$)
F_1	2.38	6.4	305	150
TF_1	1.57	4.4	238	112

F_1 was purified from bovine heart mitochondria and the hydrolase activity tested with an ATP-regenerating system in the following mixture: 5 μg F_1, 250 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 20 mM Tris, 2 mM phosphoenolpyruvate, NADH 0,1 mM, 2 units pyruvate kinase and 2.5 units lactate dehydrogenase. Final volume, 1 ml; pH 7.5; room temperature. The K_m and V_{max} values were obtained by Eadie-Hofstee plots of initial rates of ATP hydrolysis against 1–2000 μM ATP.

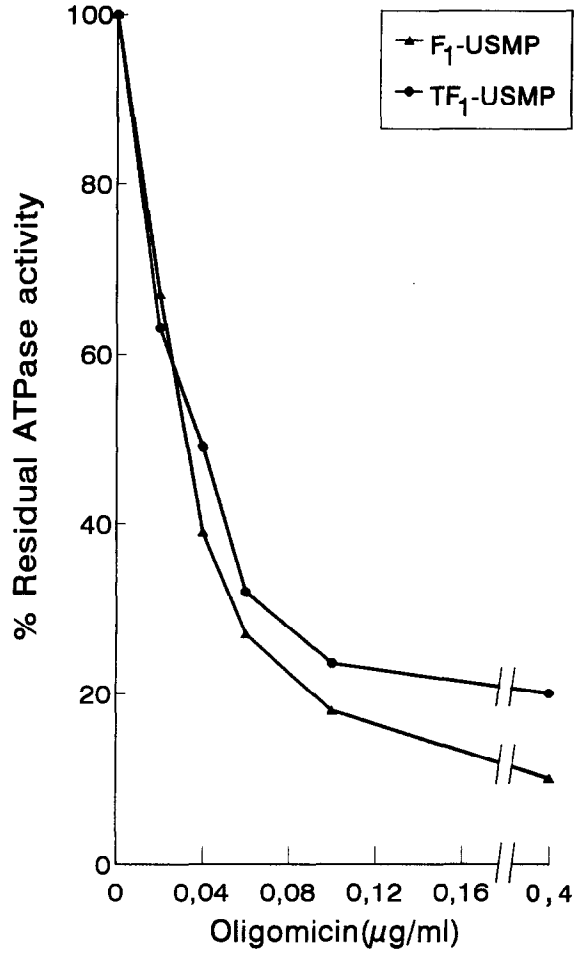

Fig. 2. Oligomycin sensitivity of ATP hydrolysis in F_1-USMP and TF_1-USMP. USMP were reconstituted with F_1 or TF_1 (10 μg protein F_1 or TF_1 per 100 μg protein USMP) and unbound F_1 or TF_1 were removed by centrifugation [17]. The pellets were suspended in 250 mM sucrose. The assay mixture for ATP hydrolysis consisted of 0.1 mg/ml USMP protein, 250 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 20 mM Tris, 2 mM phosphoenolpyruvate, 0.1 mM NADH, 2 units pyruvate kinase, 2.5 units lactate dehydrogenase and 0.5 μg rotenone; pH 7.5. Final volume, 1 ml; room temperature. The reaction was started by the addition of 1.5 mM ATP. Oligomycin titration was carried out by 2 min preincubation of the particles with the concentrations of the inhibitor reported in the figure.

substrate binding and positive cooperativity for ATP hydrolysis. Both K_{m1} and V_{max1}, were apparently decreased by trypsinolysis, however, it should be noted that the values obtained for these parameters represent only approximate estimates [26]. The K_{m2} and V_{max2}, whose estimates are more reliable, were decreased after F_1 trypsinolysis (Table 1). The decrease in both K_{m2} and V_{max2} could reflect impairment of β-α intersubunit interaction which is essential for the display of full catalytic activity of F_1 (T. Xu and S.Papa, unpublished observations). These aspects will be the subject of further study.

The effect of trypsin digestion of F_1 on its functional coupling with F_0 was studied by reconstitution of F_1 and TF_1 with USMP. After reconstitution F_1-USMP and TF_1-USMP had similar ATP hydrolase activity (V_{max} amounted to 2.8 and 2.6 μmol Pi/mg USMP protein per min respectively). The ATPase activity of F_1-USMP and TF_1-USMP showed, practically, the same sensitivity to oligomycin (Fig. 2). The I_{50} for ATPase inhibition amounted to around 0.4 μg oligomycin/mg USMP protein both with F_1-USMP and TF_1-USMP.

The ATP-driven proton pumping was followed by ACMA fluorescence quenching [24]. Compared with F_1-USMP in which ATP induced about 40% fluorescence quenching, TF_1-USMP had a 50% decreased capacity to pump protons (Fig. 3). This result is consistent with observations showing that, after trypsin digestion, F_1 lost, in part or fully, the ability to restore ATP-dependent energy coupling with USMP [11,12].

It has been shown that F_1, after reconstitution with USMP, blocks the proton channel in F_0 [23,27,28]. Following the passive, oligomycin sensitive proton conduction in USMP, which is essentially ascribed to F_0 [23], we tested the effect of trypsin digestion on the capacity of F_1 to block the proton channel in F_0. Fig. 4a shows that, after reconstitution with USMP, increasing concentrations of F_1 progressively inhibited passive proton conduction (at an F_1/USMP protein ratio of 1:12 proton conduction was inhibited by 50%). TF_1 did not produce, in the same concentration range, any inhibition of proton conduction. TF_1 appeared, rather, to result in some enhancement of proton conduction of USMP.

Previous work in this laboratory showed that F_1 ligands, for example, the nucleotide analog AMP-PNP and lipophilic cations, inhibit the passive proton conduction in F_0 [23,27]. This means that the conformational change in F_1, induced by ligand binding, can be transferred to F_0 and then affect its function. The effect of AMP-PNP on proton conduction in F_1-USMP and TF_1-USMP was, therefore, tested. Fig. 4b shows that micromolar concentrations of AMP-PNP significantly inhibited the passive proton conduction in the reconstituted F_1-USMP system. The same concentrations of AMP-

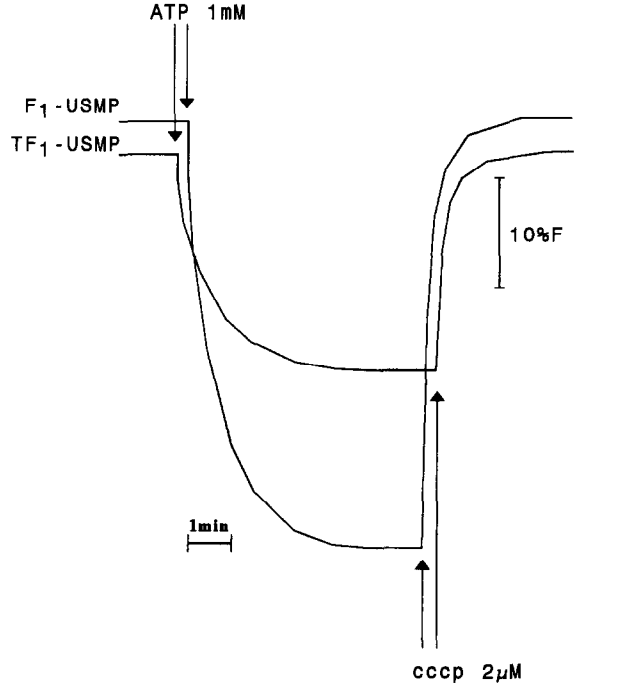

Fig. 3. ATP-driven ACMA fluorescence quenching in F_1-USMP and TF_1-USMP. Measurement was performed in 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 300 mM KCl, pH 7.4, ACMA 0.2 μg/ml, TF_1-USMP or F_1-USMP 0.1 mg/ml; room temperature. The reaction was started by the addition of 1 mM ATP. For other details see Section 2.

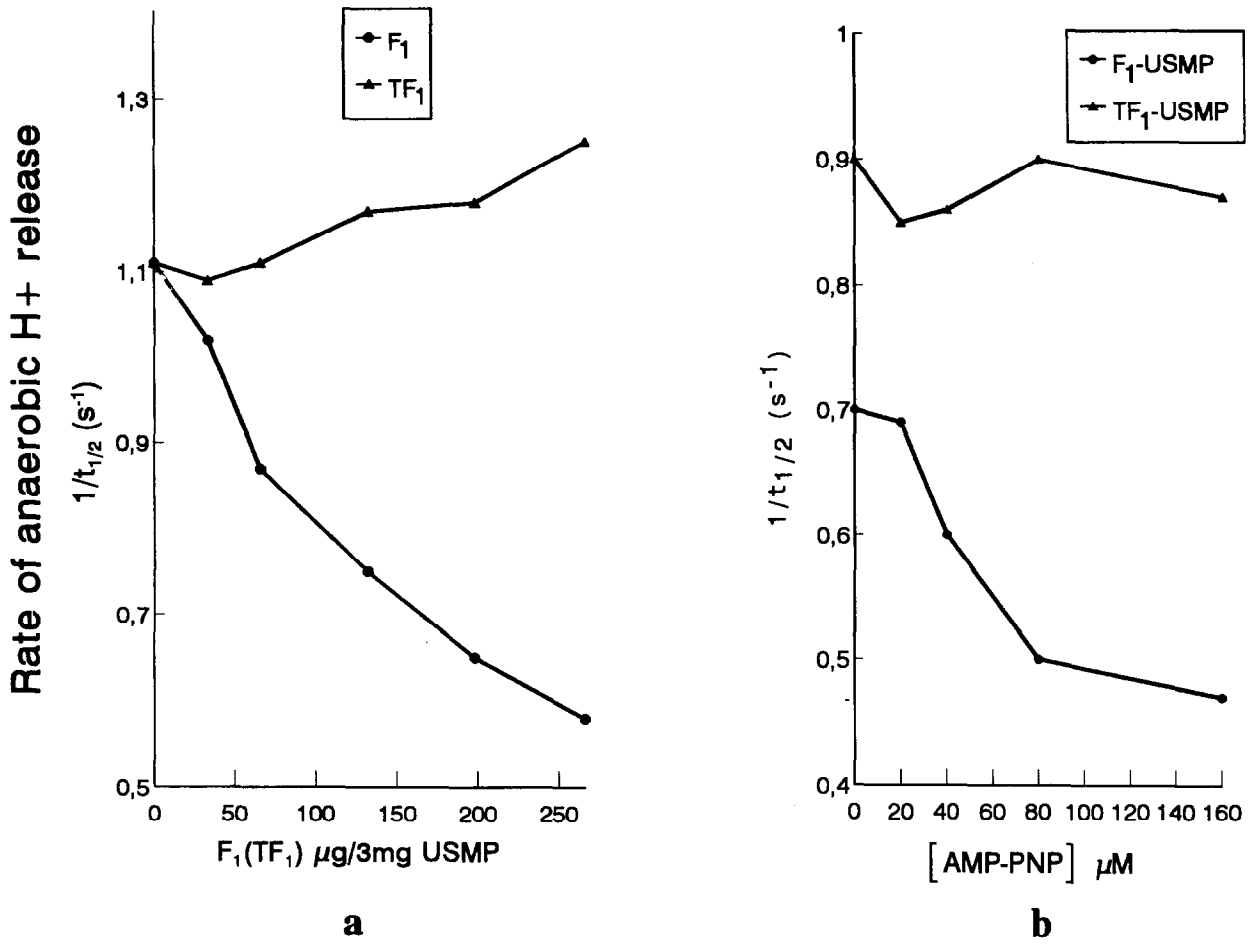

Fig. 4. Titration of the effect of F_1 and TF_1 (a) and AMP-PNP (b) on passive proton conduction in USMP. USMP were incubated under a constant stream of N_2 in 200 mM sucrose, 30 mM KCl, 2 µg valinomycin, 0.3 mg catalase, 20 mM succinate, pH 7.5 and 4.5 mg USMP protein; final volume, 1.5 ml. Respiration was activated by the addition of 5 µl 3% H_2O_2. Passive proton release ensuing upon anaerobiosis was followed potentiometrically as described in [23]. (a) 60 mM $(NH_4)_2SO_4$ was added to the mixture to enhance reconstitution. F_1 or TF_1, in the amounts specified in the figure, were added to the USMP suspension. After 10 min incubation, the respiratory pulses for measurement of proton translocation were started. (b) USMP were reconstituted with F_1 or TF_1 as described in the legend to Fig. 2. F_1-USMP and TF_1-USMP were preincubated 5 min with AMP-PNP before starting the respiratory pulses.

PNP had no inhibitory effect on the proton conduction of USMP reconstituted with TF_1. Since F_1 and TF_1 exhibited the same affinity for AMP-PNP (T. Xu and S. Papa, unpublished observations), it is not the binding of AMP-PNP to F_1 that will be affected by trypsin digestion but, rather, the transmission to F_0 of a conformational effect initiated by the AMP-PNP binding to F_1.

4. Discussion

The effects of trypsin digestion described in this paper can be ascribed to the removal of the 15-residue N-terminal segment of the F_1 α subunit (and perhaps of the first 6–7 residues of the F_1 β subunit). The sequences of the α and β subunits of bovine F_1 are weakly homologous [8]. The 15-residue N-terminal segment of the α subunit has no counterpart in the β subunit [8]. Trypsin removal of the first 15 residues from the N-terminal segment of the α subunit does not seem to impair the binding of F_1 to F_0 in USMP, nor does it affect the oligomycin sensitivity of the ATP hydrolase reaction (see also [10,11]). On the other hand, this cleavage of the α subunit does impair: (i) coupling of ATP hydrolysis with proton pumping; (ii) inhibition by F_1 of passive proton conduction in F_0; and (iii) transmission of a conformational change, initiated by the binding of AMP-PNP to F_1, to F_0 where it results in inhibition of proton conduction.

It seems as if there are two F_0F_1 interaction sites in the stalk. One contributes to the structural binding of F_1 to F_0 and to transmit the effect of oligomycin inhibition of proton conduction in F_0 to the catalytic process in F_1. The N-terminal segment of F_1 subunit α does not seem to affect this site. The other F_0F_1 interaction site in the stalk appears to be directly involved in the energy transfer between the two sectors, in particular in the coupling of the catalytic process to proton translocation and in the gating of the proton channel of F_0. The N-terminal segment of α subunit seems to be involved in this interaction. As the crystallographic X-ray analysis shows that the N-terminus of subunit α (as well as of subunit β) is located at the top of F_1 just opposite to its binding region to F_0 [7], it is difficult to visualize how this region of the α subunit could directly interact with F_0 components of the stalk. It follows that the influence of the N-terminal segment of the α subunit (and perhaps of the β subunit) on the coupling of ATP hydrolysis to proton pumping

and on the gating of proton conduction in F_0 should involve long-range conformational changes, mediated by another region of the same subunit or other F_1 subunits which are in direct contact with the F_0 subunits contributing to the stalk. In this respect, a good candidate should be represented by the F_1 γ subunit. The NH_2 and carboxy-terminal segments of this subunit form a coiled coil structure which fills up the central cavity formed by the $\alpha_3\beta_3$ hexamer, up to the top [7]. The central region of the γ subunit, on the other hand, protrudes from the bottom of F_1, constituting part of the stalk and appears to be in direct contact with hydrophilic extensions of the F_0I-PVP subunit (possible counterpart of the b subunit of *E. coli*) [20,28,29] and of the F_0-c subunit [30]. It has been shown that the γ subunit is involved in the gating of the proton channel [28]. It is possible that the N-terminal segment of the α subunit plays a role in energy-linked conformational mobility of the γ subunit. Removal of this segment of the α subunit will introduce some imbalance factors, which cause alteration in the coupling and gating function of the stalk. However, in this regard, a role of the δ/ε subunit [31] cannot be excluded.

In vitro reconstitution experiments of the stalk from isolated F_0 and F_1 subunits have been carried out in our [29,32] and other laboratories [33]. We are currently employing similar approaches to verify the role of the α subunit in energy coupling in the F_0F_1 complex and the existence of two F_0F_1 interaction regions in the stalk (a static and a mobile part?).

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