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Topological and functional aspects of the proton conductor, F_0, of the *Escherichia coli* ATP-synthase

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The isolated H^+ conductor, F_0, of the *Escherichia coli* ATP-synthase consists of three subunits, *a*, *b*, and *c*. H^+-permeable liposomes can be reconstituted with F_0 and lipids; addition of F_1-ATPase reconstitutes a functional ATP-synthase. Mutants with altered or missing F_0 subunits are defective in H^+ conduction. Thus, all three subunits are necessary for the expression of H^+ conduction. The subunits *a* and *b* contain binding sites for F_1. Computer calculations, cross-links, membrane-permeating photo-reactive labels, and proteases were used to develop tentative structural models for the individual F_0 subunits.

ATP-synthases catalyze the proton-motive-force-dependent phosphorylation of ADP in oxidative phosphorylation and photophosphorylation. Inversely, hydrolysis of ATP via the ATP-synthase is coupled to the formation of a proton-motive force. Both reactions are inhibited by dicyclohexylcarbodiimide (DCCD). The enzymes occur in remarkably conserved form in prokaryotic and eukaryotic cells. They are composed of two parts, a membrane-integrated part F_0, rendering the membrane proton-permeable, and a membrane-associated part F_1, bearing ATPase activity. The functions of F_0 and of F_0 subunits were examined by a variety of different methods: isolation of F_0, reconstitution of biological activities, isolation of F_0-mutants, elucidation of their structural alterations, and characterization of the functional implications. The structural features of individual F_0 subunits were investigated by determination of the primary structure, computer calculations of the secondary structure, cross-links, proteases, and photo-reactive labels.

Methods

The preparation of F_1, F_1F_0, F_0, membranes, F_1-depleted membranes, and antibodies against subunits was performed as described previously (Vogel & Steinhart, 1976; Friedl et al., 1979; Friedl & Schairer, 1981; Friedl et al., 1981). The constitution of strains and construction of plasmids are described elsewhere (Hansen et al., 1981; Hansen & von Meyenburg, 1980; Gunsalus et al., 1982; Friedl et al., 1981; Friedl et al., 1980); plasmid pOM 11-1 contains the structural gene of subunit *a* on a HindIII-AvaI fragment cloned in pBR322 (Michelsen O, unpublished).

Assays of F_1 binding, H^+ conduction, and ATP-dependent H^+ translocation were performed as described (Sebald et al., 1982; Friedl et al., 1980), as well as polyacrylamide-gel electrophoresis (Friedl et al., 1979) and silver-staining of gels (Merril et al., 1980). Predictions of secondary structure and membrane-permeating segments were done as described previously (Chou & Fasman, 1978; Maxfield & Scheraga, 1976; Robson & Suzuki, 1976; Nagano, 1977; von Heijne, 1981).

Proteolytic treatment of F_0-subunits in F_1-depleted membranes

F_1-depleted membranes were prepared from an *Escherichia coli* overproducer (obtained from K. von Meyenburg). Membranes (5 mg/ml) were incubated in buffer (100 mM Tris/HCl, pH 7.8, 5 mM $MgCl_2$) for 60 min at 37°C with proteases at ratios (w/w) of 1:1000, 1:100, and 1:10. After addition of protease-inhibitor phenylmethylsulfonylfluoride (PMSF) to 1 mM, membranes were washed twice with buffer and submitted to dodecylsulfate-gel electrophoresis. The protein bands were visualized by the silver-staining procedure.

Cross-linking of F_0 subunit b *from* Escherichia coli

Isolated ATP-synthase from *Escherichia coli* dissolved 1 mg per 0.2 ml in buffer (50 mM *N*-methylmorpholine, pH 8, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM PMSF, 25 mM Aminooxid WS35, 20% methanol) was incubated in the dark with 5 mM fluoronitrophenylazide for 60 min at room temperature. Thereafter the reactive nitrene was generated by illumination at 360 nm for 10 min at 0°C. Proteins were separated electrophoretically on 0.7-mm-thick dodecylsulfate gels, and were then transferred electrophoretically onto nitrocellulose sheets (Towbin et al., 1979). The sheets were incubated with specific rabbit IgG directed against subunit *b*. Bound IgG was visualized after reaction with fluorescein-conjugated goat antirabbit IgG under u.v. light.

Results and Discussion

F_1F_0 and F_0 preparations

The *Escherichia coli* ATP-synthase has been purified in large quantities by extracting membranes with the zwitterionic detergent Aminoxid WS35 and subsequent chromatography of the extract on DEAE-Sepharose (Friedl et al., 1979). SDS/gel electrophoresis of the purified enzyme revealed 8 subunits: in addition to the five subunits of F_1, α-ε, 3 polypeptides, *a*, *b*, and *c*, are found (Fig. 1), with the apparent mol. wts. of 24 000, 19 000, and 8500. After reintegration into liposomes the enzyme is active in ATP-dependent proton translocation to more than 90% (Table 1). F_0 was isolated from the purified ATP-synthase (Friedl & Schairer, 1981). Fig. 1 shows that F_0 consists of the three subunits *a*, *b*, and *c*. The catalytic activities of F_0 could be determined after integration into liposomes (Table 1). F_0 catalyzed an electro-impelled proton flux which can be inhibited by DCCD or by binding of F_1. Binding of F_1 restores the ATP-dependent proton-translocation.

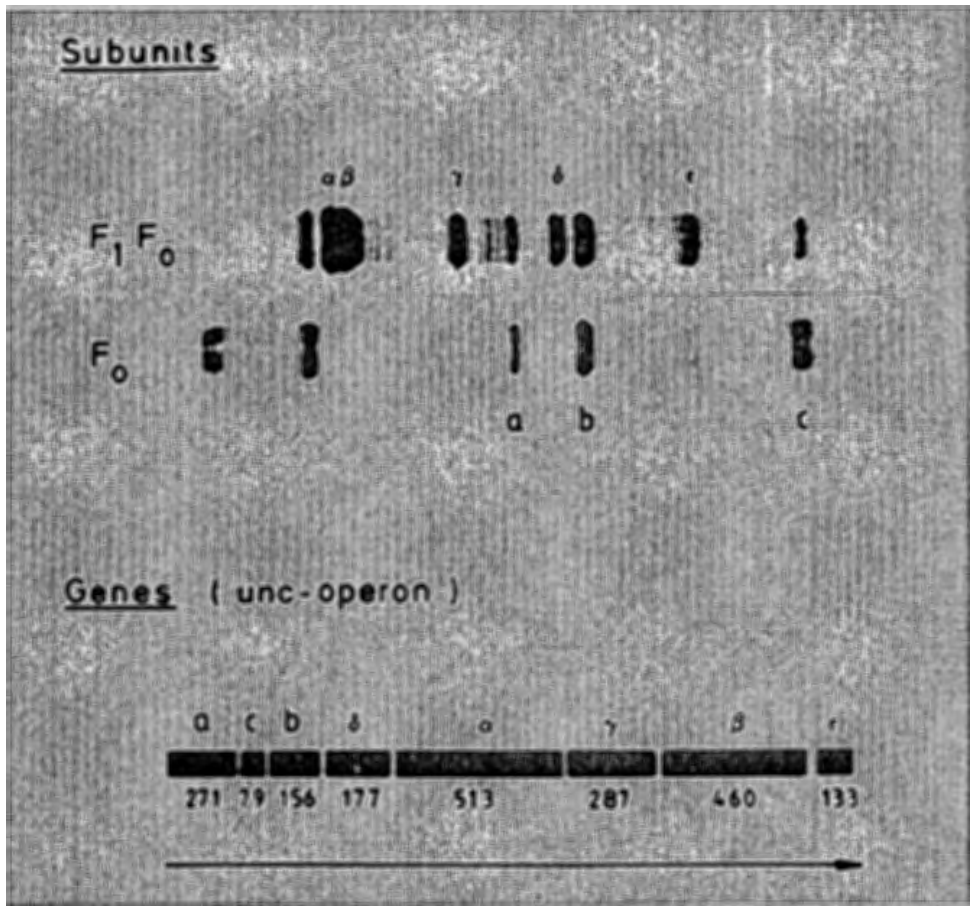

Fig. 1. Subunits and genes of the *Escherichia coli* ATP-synthase. SDS/polyacrylamide-gel electrophoresis was performed as described in methods. The order of the genes in the *unc* operon and the correlation of genes with ATP-synthase subunits was established genetically and by nucleotide sequence analysis. The numbers below the genes indicate the number of amino acid residues in the subunit polypeptides.

F_0-mutants

Genetic studies of the *E. coli* ATP-synthase started with the isolation and characterization of mutants defective in oxidative phosphorylation (for review see Downie et al., 1979). The genes coding for the polypeptides of the ATP-synthase are organized in the *unc*-operon (Fig. 1). The complete DNA sequence of the operon has been determined (Gay & Walker, 1981; Nielsen et al., 1981; Kanazawa et al., 1981). Mutants with an altered F_0-subunit *a*, *b*, or *c* are defective in proton conduction and ATP-dependent proton translocation. This indicates that in vivo all three subunits are necessary for these functions. In order to correlate certain functions to the different F_0 subunits, mutant strains were constructed lacking one or two of the F_0 subunits.

Table 1. Activities of F_1F_0 and F_0 from *Escherichia coli*

Membranes, F_1-depleted membranes, F_1F_0, F_0, and proteoliposomes were prepared and the assays were performed as described in 'Materials and Methods'.

Fraction	Electro-impelled H^+ conduction		ATP-dependent H^+ translocation
	pH-electrode	Fluorescence test	Fluorescence test
	$nmol \cdot min^{-1} \cdot mg^{-1}$	$U_{f1} \cdot mg^{-1}$	
Membranes	-	-	130
F_1-depleted membranes	44	65	-
Liposomes with			
F_1F_0	5	16	2330
$F_0 + F_1$	41	151	1910
F_0	870	2565	1
F_0 + DCCD	2	24	-

Table 2 shows that for the expression of proton conduction all three subunits of F_0 are necessary. On the other hand subunit *a* or subunit *b* alone can bind F_1, whereas subunit *c* cannot. Binding of F_1 by these subunits does not reconstitute DCCD-sensitivity of the ATPase activity or ATP-dependent proton translocation.

Table 2. Activities of F_0 in *unc*-mutants lacking individual F_0 subunits

Strain	F_0 subunits			H^+ conduction	F_1 binding
A1	a	b	c	+	+
DG10/6	a	b		-	+
CM2080 (log)		b	c	-	+
AM12	a		c	-	+
CM1470 + pOM11-1	a			-	+
CM1470 + pRPG51		b		-	+
CM2080 (stat)			c	-	-
CM 1470				-	-

Strains: A1 wild type; CM1470 and CM2080 deletion mutants; the presence of subunit *b* depends on growth phase (log = logarithmic, stat = stationary phase); AM12 and DG10/6, polar mutants; pOM11-1, plasmid with structural gene for subunit *a*; pRPG51, plasmid with structural gene for subunits *b* and δ. Tests were performed as described in 'Materials and Methods'.

Structure of F_0

The stoichiometry of F_0 seems to be unusual. Homogeneous labelling of the proteins with radioactive precursors revealed a ratio for *a:b:c* of 1:2:10 (Foster & Fillingame, 1982). The high number for subunit *c* explains the observed strong negative complementation in c^+/c^- heterozygotes (Friedl et al., 1980). But it is difficult to propose a reasonable model for F_0. The DNA sequence of the *unc*-operon combined with partial amino acid sequencing provides the primary structure of the F_0 subunits. Fig. 2 shows a computer printout for the F_0 subunits with predictions for the secondary structure (Chou & Fasman, 1978; Maxfield & Scheraga, 1976; Robson & Suzuki, 1976; Nagano, 1977) and for the membrane-permeating segments (von Heijne, 1981). The proposal for the DCCD-binding subunit *c* is the 'hairpin' model (Sebald & Hoppe, 1981) with two hydrophobic segments spanning the membrane and a central polar loop at the membrane surface. The DCCD-binding aspartyl-residue 61 is located in the lipid phase. The replacement of this amino acid by glycine or asparagine leads to an F_0 defective in H^+ conduction (Hoppe et al., 1980b; J. Hoppe, unpublished). DCCD-resistant mutants originate from substitutions of isoleucine 28 by threonine or valine (Hoppe et al., 1980a). In the hairpin model this isoleucine is located in the vicinity of the DCCD-reactive aspartyl residue. Circular-dichroism measurements support the high content of alpha-helical secondary structures of this subunit (Sebald & Hoppe, 1981).

Subunit *b* consists of 156 amino acids and is mainly hydrophilic. Only the 33 amino acids of the *N* terminus form a hydrophobic domain sticking in the membrane, the greatly alpha-helical polar moiety being exposed to the cytoplasm.

Subunit *a* is the largest F_0 subunit. The sequence of 271 amino acids shows seven hydrophobic sequences of about 20-25 residues. According to the model, they are spanning the membrane, and the *N*-terminal region of 40 amino acids could be exposed to the cytoplasm. Due to its highly hydrophobic character no protein-chemical characterization has been performed so far.

Lipophilic membrane-permeating photoreactive labels were used to modify specifically amino acid residues located in the membrane. All three subunits get labelled with trifluoromethyliodophenyldiazirine. The characteristic labelling patterns of subunits *c* and *b* support the above-presented models for these subunits (J. Hoppe, unpublished). The characterization of water-exposed segments was achieved by protease treatment of F_1-depleted membranes from an ATP-synthase-overproducing strain (obtained from K. von Meyenburg). Fig. 3 shows that subunit *c* was only marginally degraded even at high protease concentrations, and subunit *a* was only partially affected at high concentrations of subtilisin and V8 protease. In contrast subunit *b* was very protease-sensitive, indicating its accessibility from the water phase. Remarkably, chymotrypsin produced a membrane-bound cleavage product lacking 20 residues of the polar *C* terminus. Proton conduction of F_0 and binding of F_1 were not affected, but ATP-dependent proton conduction was abolished (J. Hoppe, unpublished).

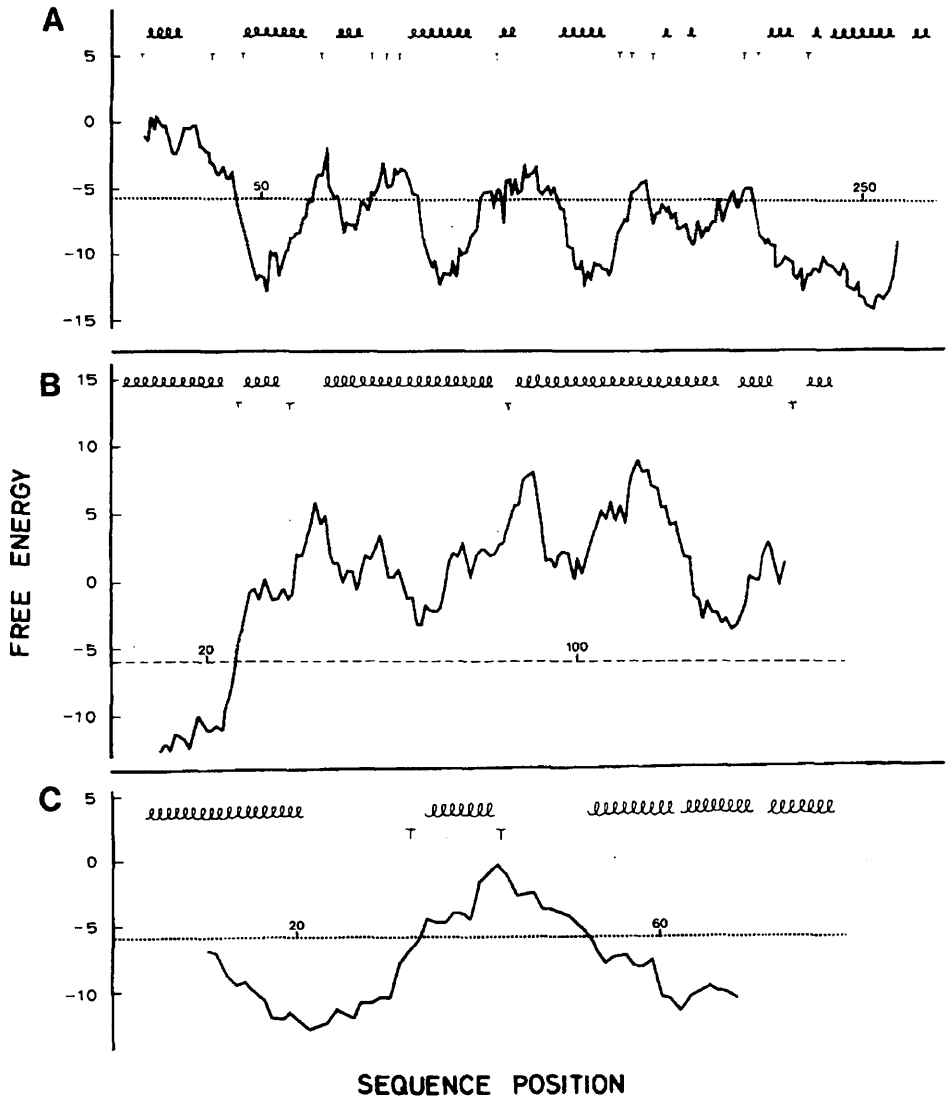

Fig. 2. Prediction of secondary structures and membrane-permeating segments in F_0 subunits of *Escherichia coli*. Alpha-helical segments (ΩΩΩ) and β-turns (T) were consistently calculated applying four different prediction methods. The free-energy gains during a transition from a random coil in water to an alpha-helix in the membranes were calculated for all amino acid sequence positions. The area below the dotted lines indicates increased probability for a location in the lipid bilayer, and the numbers on the dotted lines give the sequence position of the amino acids. Plots for subunits *a* (A), *b* (B), and *c* (C).

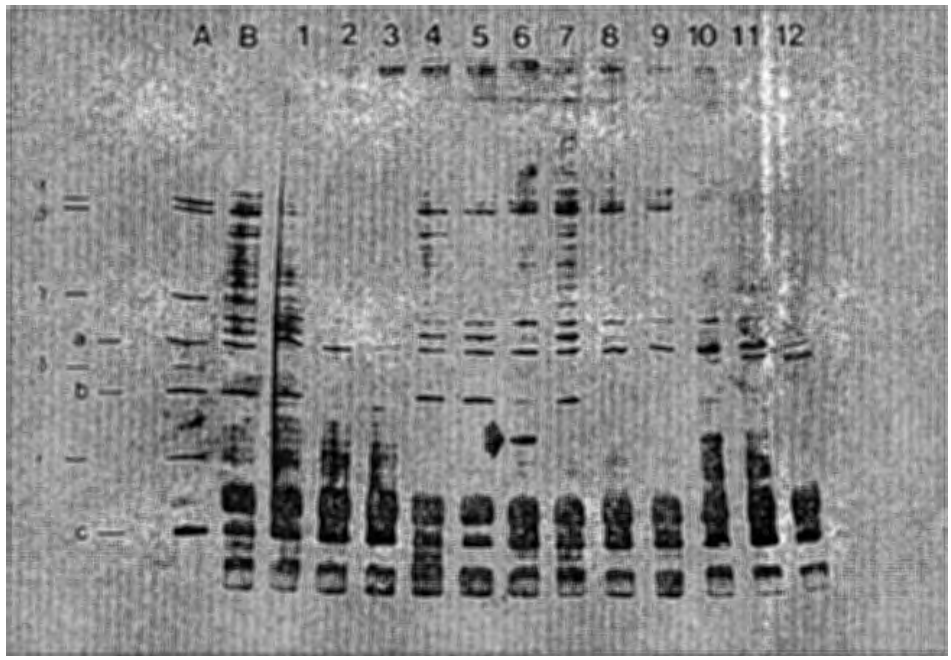

Fig. 3. Proteolytic treatment of F_0 subunits in F_1-depleted membranes. F_1-depleted membranes were prepared from an *Escherichia coli* overproducer (obtained from K. von Meyenburg). Lane A shows separated F_1F_0, and lane B, separated F_1-depleted membranes. The protease concentrations increase in the samples from left to right for V8 (lanes 1-3), chymotrypsin (4-6), trypsin (7-9), and subtilisin (10-12). The arrow indicates the digestion product obtained with chymotrypsin.

Cross-links of ATP-synthase subunits were obtained using the bivalent electrophilic and photoreactive reagent azidonitrophenylfluoride. The results of the subsequent analysis by SDS/gel electrophoresis, blotting, and immunochemical staining are shown in Fig. 4. The most prominent cross-link product is *b*-*b*, indicating a *b*-dimer in the ATP-synthase. Small amounts of additional subunit *b* cross links were observed which are tentatively assigned to cross-links with the subunits *a*, *c*, α, and β. This suggests that subunit *b* has multiple contacts with other subunits, indicating its location in the center of the ATP-synthase.

The results presented are in accordance with the prediction made for the structure of the F_0 subunits. Further elucidation of the structure of F_0 as well as of F_1 will be brought about by the use of monoclonal antibodies, peptide chemistry, and electron microscopy.

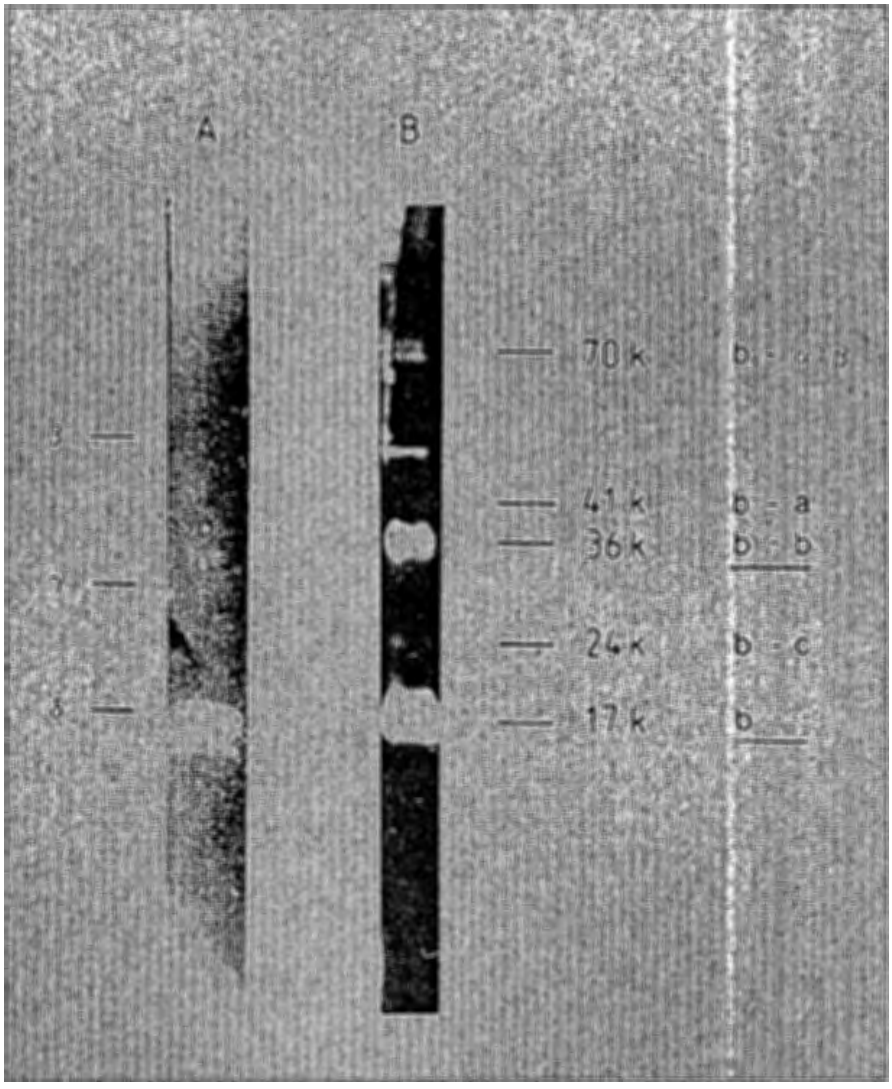

Fig. 4. Cross-linking of F_0-subunit *b* from *Escherichia coli*. Isolated F_1F_0 was cross-linked with fluoro-nitrophenylazide and subjected to SDS/polyacrylamide-gel electrophoresis, and the separated products were transferred to nitrocellulose and stained for antigenic material as described in 'Materials and Methods'. Lane A shows the identification of subunit *b* in the absence of cross-linking. Lane B demonstrates the appearance of cross-links containing subunit *b*. The positions of subunits β, γ, and δ, which were separated in parallel, are indicated.

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