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The Mitochondrial ATP Synthase of *Trypanosoma brucei*: Structure and Regulation

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The structure and regulation of the *Trypanosoma brucei* mitochondrial ATP synthase is reviewed. This enzyme complex which catalyzes the synthesis and hydrolysis of ATP within the mitochondrion is a multisubunit complex which is regulated in several ways. Several lines of evidence have shown that the ATP synthase is regulated through the life cycle of *Trypanosoma brucei*. The enzyme complex is present at maximal levels in the procyclic form where mitochondrial activity is the highest and cytochromes and Kreb's cycle components are present. The levels of the ATP synthase are decreased in the bloodstream forms where the levels of the mitochondrial cytochromes are absent or substantially decreased. In recent preliminary work we have shown the presence of an ATP synthase inhibitor peptide which may indicate an additional level of complexity to the regulation.

KEY WORDS: ATP synthase; Trypanosoma; regulation; inhibitor peptide.

INTRODUCTION

The F-type ATPase or ATP synthase has been extensively characterized from the mitochondrial inner membrane, the bacterial inner membrane, and in chloroplast thylakoids in a wide range of organisms and has been the subject of an entire minireview series in this journal (October 1992). This subgroup of the H^+-ATPases is characterized by inhibition by oligomycin and dicyclohexylcarbodiimide (DCCD) and by a higher degree of physical and functional complexity (for reviews see Futai *et al.*, 1989; Pedersen and Carafoli, 1987; Penefsky and Cross, 1991; Senior, 1990). It is comprised of two portions, F_1 and F_0 (Fig. 1) and possesses an endogenous inhibitory peptide. The F_1-ATPase, the headpiece of the enzyme complex, is hydrophilic in nature and, when isolated separately from the F_0 portion, is capable of ATP hydrolysis. The F_1 moiety, which is relatively well conserved in phylogeny, usually possesses five types of subunits in a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$. The enzyme complex possesses 4–6 nucleotide binding sites located on the larger subunits of the F_1-ATPase, α and β (Cross, 1988; Gromet-Elhanan, 1992; Senior, 1992; Williams *et al.*, 1987). The "catalytic sites" are located either on the α subunit alone or at an α/β interface (Allison, 1992; Gromet-Elhanan, 1992; Senior, 1992). The preferred substrate of the enzyme is Mg-ATP and there are a number of metal binding sites on the F_1-ATPase that are required for both structure and function (Senior, 1979; Williams *et al.*, 1987). The F_1 is connected with the F_0 by a stalk structure which appears to consist of parts of both the F_1 and F_0.

The F_0, which is membrane embedded, functions in conducting protons across the membrane. The F_0, in contrast to the F_1, is poorly conserved both in the number of subunit types present and in their primary sequence. In the best characterized example, *Escherichia coli*, there are three subunits present in the stoichiometry $a_1b_2c_{10\pm1}$ (Fillingame, 1992). In the higher eukaryotes, the subunit number is uncertain, with reports suggesting between 8 and 13 subunit types, some of which are present in multiple copies within the complex (Fillingame, 1990; Pedersen and Carafoli, 1987). The F_0 component also appears to be the site of

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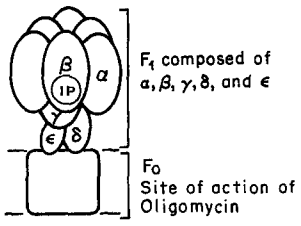

Fig. 1. The mitochondrial ATP synthase. F_1 is the soluble headpiece, F_0 is the membrane-associated portion, and IP is the endogenous inhibitor peptide.

interaction for the inhibitors oligomycin and DCCD. However, only when the complex is complete (the F_0F_1-ATPase) is it capable of coupling the protonmotive force to synthesis of ATP.

As previously mentioned, an additional component for most ATP synthases is an endogenous inhibitor peptide (Fig. 1). In most organisms there is a single endogenous inhibitor peptide, the Pullman–Monroy type inhibitor peptide, but other variations have been reported. In *E. coli* and chloroplast systems one of the F_1 subunits may act as the endogenous inhibitor. More recently, a second ATPase inhibitor peptide has been identified in bovine heart mitochondria. This calcium binding inhibitor is completely distinct from the Pullman–Monroy inhibitor (Yamada and Huzel, 1988). In yeast two stabilizing factors (15 kD and 9 kD proteins) and the ATPase inhibitor form a regulatory complex for the mitochondria ATP synthase (Hashimoto *et al.*, 1990). The Pullman–Monroy peptide inhibitor was originally shown in inhibit the ATP hydrolysis reaction (Asami *et al.*, 1970) and was subsequently shown to inhibit the ATP synthesis reaction (Tuena de Gomez-Puyou *et al.*, 1988). The inhibitor proteins bind directly to the F_1 portion of the enzyme complex, inhibiting its ATPase-hydrolyzing activity and ATPase-driven energy transfer reactions. The inhibitory activity required an acidic pH (below 6.7) and hydrolysis of the substrate (Mg-ATP) (Schwerzmann and Pedersen, 1986). Both of these requirements appear to reflect the conditions to maintain the active conformer of the inhibitor peptide (Fujii *et al.*, 1983). The inhibitor peptide has been shown to be associated with the β subunit under inhibitory conditions. In recent work Mimura *et al.* (1993) have shown that upon deenergization of mitochondria the inhibitor protein is bound to an α/β interface.

Our laboratory has begun the comprehensive characterization of the ATP synthase complex in *Trypanosoma brucei*. Our special interest is in how

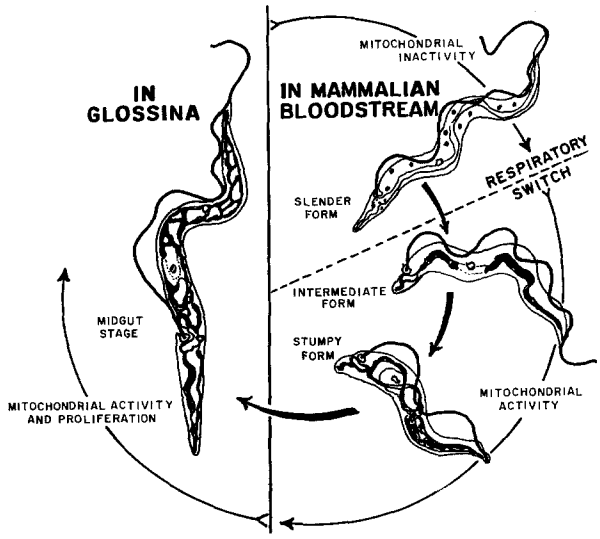

Fig. 2. The life cycle of *Trypanosoma brucei* and mitochondrial function. After Vickerman (1965).

the structure and the functional role of the ATP synthase may be altered with the radical changes that occur in the metabolism of *Trypanosoma brucei* as it shifts from one life cycle stage to another (Fig. 2). As has been known for some time, the mitochondrion of *Trypanosoma brucei* is active in oxidative phosphorylation when the parasite inhabits the gut of the insect vector (*Glossina*; see Vickerman, 1965; Bowman and Flynn, 1973). However, as it passes into the mammalian bloodstream as the insect takes a blood meal, its metabolism shifts radically to a glycolytic mode (Bienen *et al.*, 1981). The mitochondrion in this early bloodstream form shuts off its production of the electron transport chain components and Krebs cycle enzymes. Later in the mammalian host infection these components are again elevated and the mitochondrion "reactivates." Our interest is in how the ATP synthase might fit into such a scheme of mitochondrial changes. Our approach to developing an understanding of the ATP synthase was to examine the enzyme complex in detail and particularly to look at potential modes of regulation for the ATP synthase.

THE MEMBRANE-BOUND ATP SYNTHASE

The data our laboratory has gathered on the membrane-bound enzyme shows it to be similar to that of other organisms (manuscript in preparation). Electron micrographs of negatively stained inverted inner membrane vesicles (IMVs) show a surface

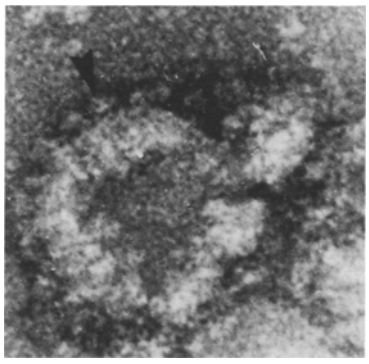

Fig. 3. Electron micrograph of the inverted inner mitochondrial membrane IMVs from the procyclic form of *Trypanosoma brucei* were negatively stained with 1% phosphotungstic acid. The arrow points to one of the F_1 headpieces that can be seen on the periphery of the inverted inner membrane vesicles. The approximate size of the headpiece is 90 Å.

studded with knoblike structures (Fig. 3) characteristic of the ATP synthase (Soper *et al.*, 1979). If the IMVs are treated with urea which removes the oligomycin-sensitive ATPase activity, the particles lose the knob structures. If purified F_1 is added back to the treated IMVs the structures are again present (Williams and Ruyechan, data not shown; see also Williams and Frank, 1990). The F_0F_1-ATPase is cold stable unlike the soluble F_1-ATPase. Oligomycin sensitivity, a hallmark of the mammalian F-type ATPases, is present although incomplete in all forms of the enzyme we have examined to date (data unpublished). These forms include purified mitochondria, submitochondrial particles, inverted inner membrane vesicles, and reconstituted inner membrane vesicles (urea-treated IMVs with F_1 added). It should be noted that although the mammalian F-type ATPases are completely inhibited by oligomycin, the *Escherichia coli* ATPase is only poorly inhibited by oligomycin (Perlin *et al.*, 1985). This may suggest a relationship of this sensitivity to the component subunits of the F_0 component, since the *Escherichia coli* system possesses the least number of F_0 subunits.

ISOLATION AND CHARACTERIZATION OF THE F_1-ATPase

The F_1-ATPase has been purified to homogeneity from the procyclic form of *Trypanosoma brucei*. The purification procedure employs chloroform extraction of inverted inner membrane vesicles prepared from mitochondria of the procyclic trypanosomes (Williams and Frank, 1990). The F_1 that is isolated by this protocol appears quite similar to that of other organisms in that there appear to be five component subunits. The estimated subunit molecular weights from SDS-polyacrylamide gel electrophoresis are 55 kD (α), 42 kD (β), 32 kD (γ), 22 kD (δ), and 17 kD (ϵ). The most notable departure from the average F-type ATPase is the size of the β subunit, which is considerably smaller than that of other organisms. Our laboratory has obtained N-terminal amino acid sequence and are now using the data to clone the gene for the β subunit and thus obtain the predicted amino acid sequence for comparison purposes (data not shown). The primary sequence for all the subunits of F_1 have been obtained from a number of sources and are available for comparison.

It is significant to note that all five subunits appear to be present in normal or near normal proportions relative to other F-type ATPases. F_1-ATPase had previously been isolated from other trypanosomatids with variable results in the subunit composition. An F_1 from *Trypanosoma cruzi* was isolated (Cataldi de Flombaum *et al.*, 1980) and showed only four component subunits (α, 62 kD, β, 54 kD, γ, 38 kD, and δ, 24 kD with an excess of δ present). *Leishmania donovani* F_1-ATPase possessed excellent activity but appears to be only partially purified (Rassam and Roberts, 1988). Higa and Cazzulo (1981) prepared F_1 from *Crithidia fasiculata* with high activity but with low levels of the ϵ subunit. The reason for this decrease in the ϵ subunit or increase in δ subunit levels may be due to the unique nature of these F_1-ATPases or it may be due to the method of isolation. The particular significance of the presence or abundance of the smaller subunits is not especially obvious since all of the preparations of F_1-ATPase from trypanosomatids appeared to have reasonable activity even in cases where they possessed abnormal subunit composition. However, the role of the smaller subunits in coupling of electron transport to ATP synthesis cannot be observed in the absence of reconstitution of the F_1-ATPase with the F_0 and subsequent analysis of either ATP synthesis or oligomycin-sensitive ATPase activity. We have shown that the F_1 isolated from *Trypanosoma brucei* by our method allows full reconstitution of oligomycin-sensitive ATPase activity and ATP synthase activity (Williams and Frank, 1990). We can state that this F_1-ATPase was fully competent for all the critical H^+-ATPase functions. We do not know whether other preparations of the F_1-ATPase from other trypanosomatids are capable of such reconstitution since

to our knowledge that was not determined. We cannot state that the absence of these smaller subunits impedes full function of these enzymes but the significance of these subunits in reconstitution has been shown in other systems such as *Escherichia coli.*

The activity of the purified *Trypanosoma brucei* ATPase appears to be quite comparable to that of other organisms with an activity in the standard Tris-HCl coupled enzyme assay with a V_{max} of 22.96 μmol/min/mg protein and a K_m value of 0.60 mM. This ATPase activity is cold labile and is not susceptible to oligomycin inhibition as is the membrane-bound enzyme. Purified ATPase reconstituted with F_1-depleted inner membrane vesicles (urea-treated particles) regains oligomycin sensitivity of its ATPase activity. The degree of oligomycin sensitivity is the same as that found in the intact inner membrane vesicles. It should be noted that we have never obtained complete oligomycin-sensitive ATPase activity in any preparation of the enzyme including mitochondria, inner membrane vesicles, submitochondrial particles, and reconstituted particles (F_1-ATPase plus urea-treated particles).

The F_1-ATPase of *Trypanosoma brucei* was analyzed for antigenic relatedness to F_1-ATPases from yeast and rat liver. The *T. brucei* ATPase showed no reactivity with the antisera to yeast or rat liver ATPases. No significant cross-reactivity was seen for the antigen and antisera for rat and yeast to the other. The *Trypanosoma brucei* antisera showed slight reactivity to the rat liver antigen (most likely the β subunit) and a slight reactivity to yeast crude cell extract in the area of the α and β subunits. Taken together, these results indicate a low level of cross-reactivity among F_1-ATPases isolated from these three eukaryotic sources.

STRUCTURE OF THE F_0 OF *TRYPANOSOMA BRUCEI*

We have only preliminary data currently available on the F_0 portion of the ATP synthase, although experiments are in progress to refine the purification of the F_0 and complete F_0F_1-ATPase. Preliminary data indicate the presence of at least 4–5 proteins comprising the F_0 component of the ATP synthase. If this proves correct after further characterization, the *Trypanosoma brucei* ATP synthase would fall below the mammalian F_0 for the number of subunits but greater than that of the *Escherichia coli* system. Many of the genes for F_0 proteins have been sequenced in other systems, but only a single putative F_0 gene from *Trypanosoma brucei* has been sequenced. This gene shows homology to the gene for yeast subunit 6 (Bhat *et al.*, 1990). This putative ATPase 6 gene (formerly MURF 4) is encoded on maxicircle DNA and its mRNA is highly edited (for a review on mitochondrial mRBA editing, see Feagin, 1990, Hajduk *et al.*, 1993, and Benne, this volume). It has been shown to be homologous (37%) to Subunit 6 by DNA sequence determination but has not been identified at the protein level. It is, however, shown to be edited in both bloodstream and procyclic forms and therefore at the level of mRNA editing is not a candidate for the regulation of the ATP synthase complex we have seen.

LIFE CYCLE-DEPENDENT REGULATION OF THE ATP SYNTHASE

All of the work performed in this laboratory on the ATP synthase has employed the *Trypanosoma brucei* strain TREU 667 for which conditions have been determined to provide the greatest proportion of the cells in a given life cycle stage (Torri and Hajduk, 1988; Michelotti and Hajduk, 1987; Rohrer *et al.*, 1987). We feel that this method, whereby a single strain is used for all experiments, has allowed us to avoid confusing results that often occur when multiple monomorphic (or polymorphic) strains are used. This is particularly true in studies designed to evaluate regulation of the ATP synthase through the *T. brucei* life cycle.

In early work Opperdoes *et al.* (1976a, 1977) showed that an oligomycin-sensitive ATPase was present in the bloodstream forms of *Trypanosoma brucei.* Our laboratory has examined our TREU 667 cells at each of the life cycle stages. We have shown by several methods that the mitochondrial ATP synthase is developmentally regulated through the *Trypanosoma brucei* life cycle (Williams *et al.*, 1991). The oligomycin-sensitive ATPase activity and the ATP synthase activity are reduced substantially in the bloodstream forms with the early bloodstream form showing the greatest reduction to less than one-third the levels in the procyclic form. Next, we examined the actual levels of ATP synthase protein present in each of the life cycle stages by Western blot analysis. Antibodies were developed separately against the F_1 and F_0 moieties purified from *T. brucei.* The Western blots of the procyclic form showed strong reactivity with

both the F_0 and F_1 antibodies. The other two life cycle stages, the early and the late bloodstream forms, showed considerably less reactivity, with the early bloodstream form being the least reactive. These results were in concurrence with our activity results. We have also examined electron micrographs of the sonicated mitochondrial fraction (as well as purified inner membrane vesicles, unpublished data). These micrographs clearly show that the degree of studding of the vesicle surface correlates to the results obtained by activity and antibody reactivity. The inverted vesicles are heavily studded with knobby ATP synthase molecules in the procyclic form, but are considerably less studded in the bloodstream forms with the very little studding present in the early bloodstream form.

KINETIC REGULATION OF THE ATP SYNTHASE

Most recently, we have begun to examine the *Trypanosoma brucei* ATP synthase for another form of regulation. As mentioned in the introduction, most F-type ATPases possess an inhibitor peptide. The presence of such an inhibitor peptide in *Trypanosoma brucei* was unclear until recently. Opperdoes *et al.* (1976b) performed a heterologous experiment in which the PMI beef heart inhibitor was added to submitochondrial particles from *Crithidia luciliae* and no inhibition was obtained. The authors felt that either there was no homologous protein in the trypanosome or (more probably) that the two proteins were simply too different for the heterologous experiment to work. Other authors have cited this work to suggest (without additional data) that no inhibitor existed (Nolan and Voorheis, 1992). A similar heterlogous experiment was reported for *Crithidia fasiculata* (Yarlett and Lloyd, 1981) with the same negative results. Some suggestion of the presence of an inhibitor peptide from *Crithidia* was seen in a "heat-treated cell-free extract" although no further purificiation or characterization was obtained.

We have recently begun work to determine whether such an inhibitory peptide does exist in *Trypanosoma brucei.* In work to be published we have purified an endogenous inhibitor by modification of procedures for isolation of a eukaryotic inhibitor (Pullman and Monroy, 1963; Cintron and Pedersen, 1979). This peptide has an approximate molecular weight of 12–15 kD and appears to be a potent inhibitor of both the trypanosomal and the rat liver mitochondrial ATPase. The cross-inhibition we see is in contrast to the results of the heterologous experiment of Opperdoes *et al.* (1976b) and our own results with the rat liver inhibitor peptide and trypanosomal IMVs. We find that this inhibitor peptide appears to be released in the preparation of inverted inner mitochondrial membrane vesicles (and submitochondrial particles) as was thought to be the case of the rat liver IMV preparation (data not shown).

We are currently trying to determine whether our inhibitor peptide is regulated through the life cycle as is the ATP synthase. In preliminary experiments we find that the inhibitor is not down regulated in the early bloodstream form but may actually increase. If these results are verified, some very interesting conclusions are possible on the role of the inhibitor peptide and the function of the ATP synthase in the lifecycle of *Trypanosoma brucei.*

PHYSIOLOGICAL ROLE OF THE ATP SYNTHASE

We do not yet understand the role or roles of the ATP synthase through the *T. brucei* life cycle. However, a careful examination of the ATP synthase begins to reveal several important pieces to a complex puzzle involving the relationship of *Trypanosoma brucei* to its environment. The absence of cytochromes in the mammalian vector form has been demonstrated and several laboratories (Benne *et al.*, 1986; Feagin and Stuart, 1988; Michelotti and Hajduk, 1987) have undertaken analysis of the regulation of expression of these proteins. Mitochondrial respiration in bloodstream trypomastigotes appears to occur through an alternative oxidase that is salicylhydroxamate sensitive (Bienen *et al.*, 1991). The authors suggest that the transitional stages of the bloodstream form generate a protonmotive force and may generate ATP at Site I of the electron transport chain (NADH dehydrogenase). Nolan and Voorheis (1990, 1992) have measured the bloodstream form for maintenance of a protonmotive force as well. They find a protonmotive force in all bloodstream forms and suggest that it is generated by the ATP synthase acting in the ATP hydrolytic mode, similar in fashion to its function in *Escherichia coli* under anaerobic conditions. The function of the ATP synthase in either maintenance of a protonmotive force by ATP hydrolysis as suggested by Nolan and Voorheis or in coupling to the protonmotive force generated by NADH dehydrogenase using

a terminal trypanosomal alternate oxidase would appear to require delicate regulation. An excess of ATP hydrolysis in either case would lead to the loss of any ATP generated by either the ATP synthase itself or by substrate level phosphorylation. We believe that the developmental regulation we have demonstrated in addition to the potential role of the inhibitor peptide may be important components of the regulation of the ATP synthase in this complex metabolic balance.

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