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Viscosimetric analysis of the mechanism of ATP hydrolysis by pea chloroplast F_1-ATPase

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Abstract

Dependence of ATP hydrolysis kinetics by the chloroplast coupling factor (CF_1) on medium viscosity was studied at varying temperatures. For samples with oxidized and reduced CF_1 γ-subunit, this dependence was shown to be described by Cramers' relationship $k \sim (\eta/\eta_o)^{-n}$, where k is the reaction rate constant, η/η_o is the medium/water viscosity ratio, and $0 < n < 1$. Transition of the γ-subunit from its reduced to oxidized state was accompanied by increasing n value, which is indicative of increasing friction losses between certain enzyme sections and the solution. The increased medium viscosity produced no effect on the reaction activation energy which appeared to be almost the same for the both enzyme states. The molecular mechanisms responsible for CF_1 activity loss in viscous media are discussed.

Additional keywords: ATP hydrolysis; F_1-ATPase; kinetics; viscosity.

Introduction

The chloroplast F_1-ATPase (CF_1) is a stroma-exposed catalytic part of the enzymatic complex CF_oF_1 that synthesizes ATP from ADP and phosphate using energy of electrochemical transmembrane potential of protons. CF_1 extracted into aqueous phase exhibits ATPase activity. The aqueous medium typically used for finding kinetic parameters of ATP synthesis/hydrolysis has a viscosity close to that of water (1 cP at 20°C). According to indirect estimates, chloroplast stroma viscosity is much higher (Ellis 1979, Asada 2004, Wayne 2009), which may result in diffusion-caused inhibition of the reaction, thereby making its *in vivo* kinetic parameters differ much from those reported in the literature (Kartashov *et al.* 2015, Malyan 2016). Also, as exemplified by a number of enzymes, high viscosity is capable of suppressing enzyme activity, provided that a part of energy required for conformational changes of an enzyme molecule during catalysis is spent on medium friction (Kramers 1940, Puchkov 2013). Therefore, knowledge of the viscosity effect would not only elucidate the difference between kinetic parameters of the reaction in question *in vitro* and *in vivo* but also shed light on the catalytic mechanism involved. This issue is of special interest because the functional mechanisms of chloroplast, bacterial, and mitochondrial F_1-ATPases differ dramatically from enzymes so far subjected to the viscosimetric analysis. According to modern concepts, F_1-ATPases may be regarded as rotary engines, where chemical energy released during ATP hydrolysis at catalytic sites located at the interface between alternating 3α- and 3β-subunits, which act as a stator, turns into mechanical energy of rotation of γ- and ε-subunits (for the F_1-ATPase structure, *see*, *e.g.*, review of Nakanishi-Matsui *et al.* (2016). The rotation is interrupted by dwells corresponding to the steps of substrate binding, its catalytic conversion, and dissociation of reaction products (Noji *et al.* 2017). In the course of ATP hydrolysis, F_1-ATPases undergo considerable conformational changes including the mentioned rotation of γ- and ε-subunits, an essential rearrangement of the active site domain, and reciprocal motions of the β-subunit C-terminus (Abrahams *et al.* 1994, Nakanishi-Matsui *et al.* 2016). The issue of the efficiency of F_1-ATPase-induced energy conversion is currently in the focus of discussion in the literature. Some authors

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Abbreviations: CF_1 – chloroplast coupling factor 1; CF_{ox} and CF_{red} – CF_1 preparations with oxidized or reduced C199–C205 bridge at the γ-subunit; E – activation energy, kcal mol^{-1}; k – reaction rate constant; K_m – apparent Michaelis constant; MgATP – equimolecular mixture of $MgCl_2$ with ATP; V_{max} – maximum reaction rate; T – absolute temperature in °K; η and η_o – viscosity values of solution and solvent in cP.
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conclude that they are 100% efficient (Yasuda *et al.* 1998, Kinosita *et al.* 2000, Itoh *et al.* 2004, Toyabe *et al.* 2011, Soga *et al.* 2017), while others point to drawbacks in interpretation of the experimental data, thus evaluating the F_1-ATPase functioning efficiency lower than 100% (Martin *et al*. 2014, Chapman and Loiselle 2016, Kulish *et al.* 2016). The theoretical studies by Kramers and others provide the basis for a qualitative estimate of energy loss resulting from protein–medium interaction in the course of reaction-induced conformational rearrangements of the protein molecule using the empirical coefficient *n* from the relationship:

$$k \sim (\eta/\eta_o)^{-n} \quad (1)$$

where *k* is the reaction rate constant, η and η_o are viscosity values of a solution and solvent, respectively, $0 < n < 1$ (Kramers 1940, Sitnitsky 2010). The larger *n*, the higher the inhibitory effect of medium viscosity on protein conformational rearrangements (Uribe and Sampedro 2003, Barbier and Campbell 2005). According to this theory, the activity decrease observed in viscous solutions results from increasing frictional losses between sites of the enzyme undergoing conformational rearrangements and molecules of the medium, which lead to an increase in the activation energy of the reaction (Khoshtariya and Goguadze 1986). However, it should be noted that recent studies of viscosity dependence of enzyme reaction rates give grounds for questioning the universality of the relationship (1) and the mentioned limits of variation of the viscosity exponent. Sashi and Bhuyan (2015) report that this dependence is determined by many interdependent factors, including both mode and consequences of protein–solvent interactions. To study the viscosity dependence of enzyme reaction kinetics, substances modulating viscosity of aqueous solutions (viscosigens) are used. Single-molecule measurements of F1-ATPase in *E. coli* (Spatzler *et al.* 2009) revealed that a polyethylene glycol (PEG-400)-induced increase of medium viscosity lengthens dwells in γ-subunit rotation coupled with the stages of ATP binding and conversion. With polyethylene glycol, dextran, and sucrose acting as viscosigens, we found that their limited concentrations increased Michaelis constant of the photophosphorylation reaction without affecting its maximum rate, which was indicative of the diffusion nature of reaction inhibition (Kartashov *et al.* 2015). Further enhanced viscosigen concentrations with accordingly increased medium viscosity gave lower maximum rates of photophosphorylation, but interpretation of these results was impeded by the evolving uncoupling and inhibition of electron transport, as well as structural heterogeneity of the chloroplasts. These difficulties could be avoided when using homogeneous aqueous solutions of the coupling factor CF_1 exhibiting ATPase activity. ATPase properties of CF_1 from spinach chloroplasts were maintained by its latent-to-active state transition due to reduction of the disulfide bridge (C199–C205) in the γ-subunit (Nalin and McCarty 1984) and addition of sulfite and ethanol to the reaction medium [for details *see* Malyan (2016) and references therein]. Similar to the viscosity influence on the photophosphorylation rate, the Michaelis constant was observed to grow after sucrose addition to the medium, which is indicative of a diffusion character of reaction inhibition at low substrate concentrations. It was also found that the use of an enzyme containing oxidized dithiol (C199–C205) resulted in a different kind of dependence, that is, with decreasing maximum reaction rate, the Michaelis constant remained virtually unchanged. The current study was aimed to elucidate the mechanisms responsible for altered kinetics of ATP hydrolysis in viscous reaction mixtures over a wide range of viscosity and temperature. In experiment, we used pea chloroplast coupling factor displaying a high ATPase activity. For samples with oxidized and reduced γ-subunit, further referred to as CF_{ox} and CF_{red}, the obtained dependencies were shown to be described by the relationship (1); importantly, the exponential factor was higher for CF_{ox}. An increase in medium viscosity produced no effect on the activation energy of reaction catalyzed by either form of the coupling factor; the activation energy values of the both forms were found to be close to each other. The mechanisms responsible for CF_1-ATPase activity loss in viscous media are discussed.

Materials and methods

The coupling factor CF_1 was isolated from pea chloroplasts according to Binder *et al.* (1978) and stored in 2 M ammonium sulfate in the presence of 1 mM ATP, 1 mM EDTA, and 50 mM Tris-SO_4, pH 7.8. Excess nucleotides and ammonium sulfate were removed by gel filtration through a *Fine Sephadex G-50* column equilibrated with 50 mM Tricine-KOH, pH 7.8. To unmask ATPase activity, CF_1 was incubated with 50 mM dithiothreitol for 2 h at room temperature. Protein concentration was determined according to Bradford (1976). The ATP hydrolysis rate was determined by HPLC. For this purpose, 15 μl reaction mixture was loaded onto a 0.5 × 7.5 DEAE 5PW column equilibrated with 120 mM NaCl and 80 mM KH_2PO_4 (pH 4.0), followed by calculation of ADP and ATP amounts from the appropriate peaks. The reaction was performed in a constant-temperature vessel with magnetic stirring at 25ºC in 0.5 ml of the reaction mixture containing 50 mM Tricine-KOH (pH 7.8), 0.3 mM $MgCl_2$, 75 mM K_2SO_3, 10% (v) C_2H_5OH, 1.5–3.0 μg(CF_1) ml^{-1}, MgATP and sucrose concentrations as indicated in the figures. Incubation time (5–10 min) and protein concentrations were chosen so that ATP conversion did not exceed 10%. Equimolecular mixture of $MgCl_2$ with ATP was used as a reaction substrate. The match between viscosity and mass

fraction of sucrose was determined using reference data (Asadi 2005). Graph approximation and calculation of kinetic parameters were performed using *Origin 6*.

Results

In order to elucidate the viscosity effect on kinetic parameters of ATP hydrolysis, changes in the maximum reaction rate (V_{max}, showing enzymatic activity) and in the apparent Michaelis constant (K_m) were estimated upon addition of sucrose to the reaction mixture. An analysis of dependencies shown in Fig. 1 revealed that with 31% sucrose concentration corresponding to viscosity of 3.0 cP (Asadi 2005), V_{max} of ATP hydrolysis by CF_{red} decreased from 0.34 ± 0.01 to 0.23 ± 0.01 μmol s^{-1} mg^{-1}(protein). The value of K_m increased from 0.57 ± 0.07 to 0.79 ± 0.07 mM. The CF_{ox} samples showed a lower maximum reaction rate (0.22 ± 0.01 μmol s^{-1} mg^{-1}) that further decreased to 0.13 ± 0.008 μmol s^{-1} mg^{-1} after sucrose addition to the mixture, while K_m remained practically unchanged (0.51 ± 0.09 mM). To reduce the experimental error, hydrolysis rates obtained at MgATP concentrations exceeding the 5–8-fold K_m were taken in subsequent experiments as the maximum reaction rates.

Fig. 2*A* shows sucrose concentration dependency of the rates of reactions catalyzed by CF_{red} and CF_{ox}. For CF_{red} over a sucrose concentration range of 5–20%, a decrease of the reaction rate never reached 10%, thus corresponding to a moderate viscosity of the medium (< 1.7 cP). With sucrose concentration increased to 40%, the viscosity level was much higher, thus causing an essential decrease in the reaction rate. This effect was more prominent in case of CF_{ox} as compared to CF_{red}. An analysis of the results presented in the ln V_{max} – ln η coordinates (Fig. 2*B*) shows that the dependency is described by the relationship (1); it is of note that the term n is higher for CF_{ox} than that for CF_{red} (0.54 and 0.34, respectively).

To address the issue of viscosity dependence of ATP hydrolysis activation energy, temperature dependence of the reaction rate was studied (Fig. 3). The data from Asadi (2005) were used to select sucrose concentrations that allow unchanged viscosity of 3 cP at increasing temperature. Activation energy (E) calculated from the Arrhenius equation

$$k = A\, e^{-E/RT} \qquad (2)$$

for linear sections of the curves in the absence and presence of sucrose amounted to 19.5 and 19.4 kcal mol^{-1}, respectively, in case of CF_{red}, and 20.1 and 19.3 kcal mol^{-1} in case of CF_{ox}., *i.e.*, within the experimental error they remained practically constant. Thus, the viscosity level increasing within 3 cP did not affect the activation energy, irrespective of the oxidized or reduced state of the CF_1 γ-subunit.

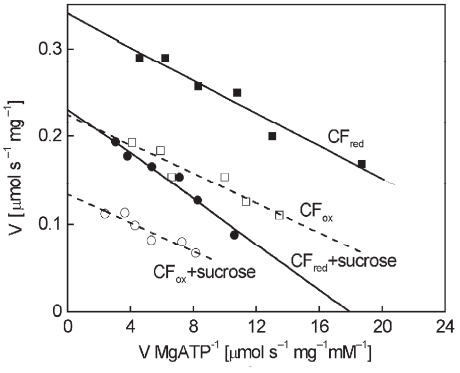

Fig. 1. Eadie-Hofstee graph presenting dependence of the ATP hydrolysis rate on MgATP concentration without or with 31% sucrose for reduced (CF_{red}) and oxidized (CF_{ox}) forms of CF 1. V – reaction rate. For reaction conditions *see* Materials and methods section.

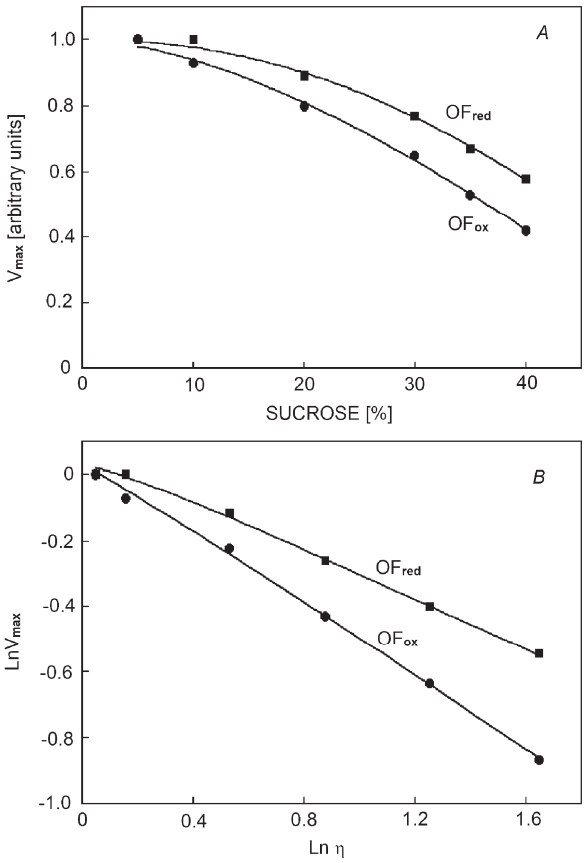

Fig. 2. Dependence of the maximum rate of ATP hydrolysis (V_{max}) catalyzed by reduced/oxidized CF 1 on (*A*) sucrose concentration and (*B*) medium viscosity (logarithmic coordinates). Reaction rates of 0.34 μmol s^{-1} mg^{-1} and 0.22 μmol min^{-1} mg^{-1} for CF_{red} and CF_{ox}, respectively, were taken to be equal to unity.

Discussion

The fact that the apparent Michaelis constant increased with increasing medium viscosity in experiments on reduced CF_1 (CF_{red}, Fig. 1) is consistent with the results previously obtained for the similar enzyme from spinach chloroplasts (Malyan 2016) that can be explained by overcoming the diffusion effects by elevated MgATP concentrations. The activity decrease (reduced V_{max}) as well as the value of n = 0.34 are apparently caused by impeded structural rearrangements of the functioning enzyme in a viscous mixture and agree with the supposition (Martin *et al*. 2014, Chapman and Loiselle 2016, Kulish *et al.* 2016) that the CF_1 effectiveness is below 100%. According to modern concepts (Abrahams *et al.* 1994, Martin *et al.* 2014, Noji *et al.* 2017), ATP binding to the catalytic site of one of the β-subunits makes its C-terminal loop shift towards this site and push the γ-subunit, thus causing its rotation. Simultaneously, the C-terminal loop of another β-subunit moves in the opposite direction, which is accompanied by dissociation of reaction products. At the catalytic site of the third β-subunit ATP turns into ADP plus phosphate. Formally speaking, the absence of notable changes in activation energy under conditions of elevated viscosity (Fig. 3) indicates that enzymatic activity is reduced due to a lower pre-exponential factor in the Arrhenius equation which, in turn, might be provoked by longer dwells in γ-subunit rotation at the steps of substrate binding and converting in conditions of growing medium viscosity, similar to what was reported for *E. coli* F_1-ATPase by Spetzler *et al.* (2009). According to the proposed interpretation of Kramer's theory (1940), the absence of activation energy changes suggests that conformational changes of the catalytic site domain at the step of conversion of tightly bound ATP into ADP and phosphate are unrestricted by medium viscosity. Then, the CF_1-ATPase under study differs from both myosin ATPase and E_1E_2-ATPase of *Kluyveromyces lactis* plasma membrane whose activation energy increases with increasing medium viscosity (Khoshtariya and Goguadze 1986, Sampedro and Uribe 2004).

The absence of K_m alteration during the CF_{ox}-induced catalysis shows that, in contrast to CF_{red} catalysis, the reaction rate is unaffected by diffusion induced inhibition. This is a well-expected result because the CF_{ox} activity is approximately two times lower than that of CF_{red} (Fig. 1). An increase of the empirical factor n (Fig. 2*B*) may result from increased friction losses between certain enzyme sections and the solution. According to Konno *et al.* (2012), the oxidized and reduced states of the enzyme

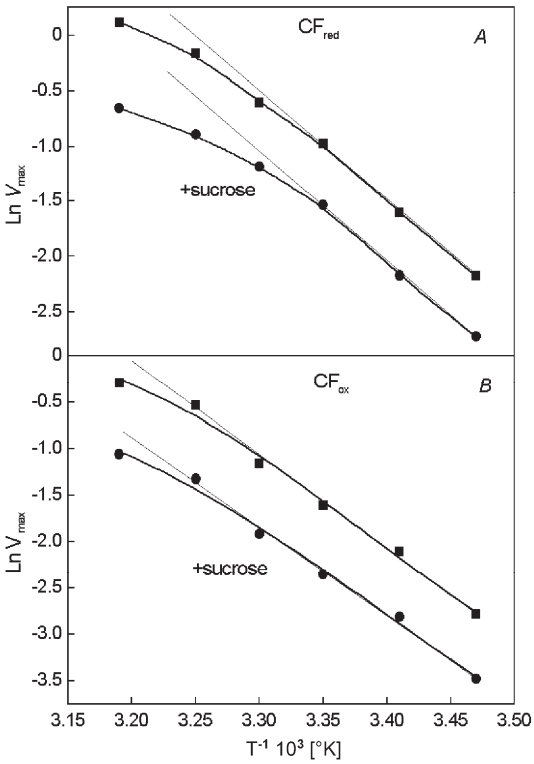

Fig. 3. Medium viscosity independent activation energy of ATP hydrolysis catalyzed by reduced (*A*)/oxidized (*B*) forms of CF 1. The sucrose concentrations at each temperature that allow unchanged viscosity of 3 cP selected from Table A12 of Asadi (2005) were as follows: 26% sucrose at 15°C, 29% at 20°C, 31.5% at 25°C, 34% at 30°C, 36.5% at 35°C, 39% at 40°C. T – absolute temperature; LnV_{max} – natural logarithm of the maximum reaction rate.

differ from each other by behavior of its ε-subunit. In F_1-ATPases of different origins, the state of this subunit may be either "down" or "up", the latter meaning that the ε-subunit C-terminus is embedded between the stator α-, β-subunits and the double α-helix of the rotor γ-subunit, thus preventing rotation of this subunit and making the enzyme inactive (Konno *et al.* 2012, Nakanishi-Matsui *et al.* 2016). It was shown that frequency of down-to-up transitions of the ε-subunit and duration of its keeping the up state (which in turn determines frequency and duration of dwells in rotor rotation) increase after enzyme oxidation. This allows suggestion that an increase of the exponent n in relationship (1) results from more frequent structural rearrangements of the ε-subunit, which is characteristic of the enzyme with oxidized γ-subunit.

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