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Kinetic analysis of proton translocation catalyzed by F_0F_1 ATPase

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Kinetic analysis of both proton translocating and steady-state ATP hydrolytic activities catalyzed by F_0F_1 ATPase in submitochondrial particles were carried out over an ATP concentration range of 1–2000 μM. The results were examined in relation to the prediction based on the alternate binding change model proposed by Gresser et al. [(1982) J. Biol. Chem. 257, 12030–12038] in which energy transduction occurs only at the tri-site catalytic cycle. The present results essentially contrast with the model and rather indicate that if the alternate binding mechanism holds for the ATP hydrolytic reaction, the proton translocation should be coupled to at least both bi-site and tri-site cycles.

F_0F_1-ATPase; Proton translocation; Alternate binding change model; Negative cooperativity; (Submitochondrial particle)

1. INTRODUCTION

Coupling of proton translocation to ATP hydrolytic reaction by F_0F_1 ATPase is one of the central problems in bioenergetics. Among many hypotheses on the mechanism of F_0F_1 ATPase, the alternate binding change model proposed by Boyer's group [1] seems most consistent and convincing. According to their model, the enzyme carries three catalytic sites and the product release occurs from singly occupied enzyme, E_D (single site catalysis), doubly occupied enzyme, E_{DT} (bi-site catalysis), and triply occupied enzyme, E_{DTD} (tri-site catalysis). During the catalysis, the proton translocation is coupled to the step of transition between E^*_{DTD} and E_{DTD} which was denoted as the 'binding change' [1]. Thus, the kinetics of proton translocation should be in parallel with that of the tri-site catalysis.

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Abbreviations: SMP, submitochondrial particle; ANS, 8-anilino-naphthalene-1-sulfonate; E_D, an enzyme which binds ADP and P_i; E_{DT}, an enzyme which binds ATP, ADP and P_i; E_{DTD}, an enzyme which binds two ADPs, P_is and ATP; E^*_{DTD}, the energized form of E_{DTD}

It is well known that the ATP hydrolytic activity of the enzyme shows a negative cooperativity [1–3], however, the kinetics of proton translocation has not been elucidated in a sufficiently wide range of substrate concentration. Although it has not been directly shown by the authors, calculations applying the parameters and equations in [1] predict that the tri-site catalysis, essentially proton translocation, would reveal a positive cooperativity in the substrate range of 1–2000 μM (see fig.2B in this text) in contrast to ATP hydrolysis. Therefore, in order to test the scheme, it is critical to examine and compare the kinetics of both the proton translocation and ATP hydrolysis reaction in a wide substrate concentration range.

In the present study, we studied the kinetics of proton translocation using submitochondrial particles in a substrate concentration between 1 and 2000 μM. This was sufficient to analyze the cooperative behavior and compared it to that of the ATP hydrolytic reaction under similar conditions. It was demonstrated that in contrast to the expectation described above, the kinetics of proton translocation also revealed a negative cooperativity with K_m values similar to those of ATP hydrolytic reaction. We also discuss the present results in relation to other hypotheses.

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2. EXPERIMENTAL

SMPs were prepared from beef heart mitochondria suspended in 0.25 M sucrose, 10 mM Tris-Cl (pH 7.5), 5 mM $MgSO_4$, by sonication followed by centrifugation [6]. ANS fluorescence measurements were performed as described [4,5]. The assay mixture contained 50 mM Tris-SO_4 (pH 8.0), 2 mM $MgSO_4$, 7 mM KCN, 5 mM phospho*enol*pyruvate, 3 μM ANS and 29 μg of pyruvate kinase in 2 ml. Typically, SMPs of 300 μg protein were used per assay. The steady-state ATP hydrolysis was measured spectrophotometrically by coupling to the oxidation of NADH [7] under similar conditions in the presence of 0.32 mM NADH, 54 μg lactate dehydrogenase, 0.54 μg FCCP and without ANS in 1.8 ml of assay mixture. Typically, SMPs of 50 μg protein were used per assay. Oligomycin sensitivity (1 μg/ml) was constant (98.5 ± 2%) over the ATP concentration range examined. The temperature was controlled to 24.5°C throughout the measurements.

The Michaelis constants were calculated by a non-linear least square method after plotting the experimental data in the form of both a Lineweaver-Burk plot and a Hanes-Woolf plot, and averaged. The V_{max} values were then adjusted to fit in an Eadie Hofstee plot using the averaged K_m values. The simulation lines in fig.2 were calculated using the kinetic parameters and equations described by Gresser et al. [1] in the ATP concentration range of 1–2000 μM.

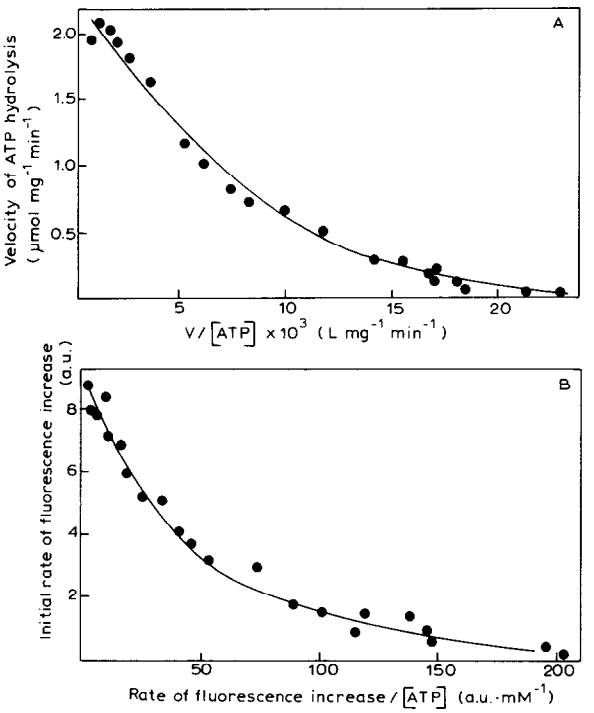

Fig.1. Eadie Hofstee plots of the ATP hydrolysis reaction (A), and the initial rate of fluorescence enhancement (B).

3. RESULTS AND DISCUSSION

3.1. *Negative cooperativity of ATPase and proton translocation*

In fig.1A, an Eadie Hofstee plot of typical data from the ATPase reaction is shown. The shape of the curve is concave and thus it is obvious that there exists a typical negative cooperativity. The high K_m and low K_m values were calculated to be 220 ± 50 and 11 ± 6 μM (n = 10), respectively. The slight differences from the values previously described by others [1,2] may be due to either the different experimental conditions or preparations such as an isolated F_1 used in their studies. The solid line in fig.1A is the theoretical curve providing two K_m values of 220 and 11 μM.

Fig.1B is an Eadie Hofstee plot of typical data from the ANS fluorescence measurement. The shape of the curve clearly reveals the negative cooperativity of the proton translocating activity. The two K_m values were calculated to be 200 ± 50 and 9 ± 3 μM (n = 6), respectively, which coincided very well with those of the ATPase activity described above. The solid line in fig.1B is the theoretical curve. From these results, it is concluded that at ATP concentrations of 1–2000 μM, both ATPase and proton translocating activities show a negative cooperativity with the same K_m values.

The use of SMPs in this type of experiment seems to be essential, since, in many cases, purified enzymes manifest unknown lag times in ATPase activity [8,9]. In fact, we found that the initial rates of fluorescence enhancement are significantly underestimated particularly at low ATP concentrations in reconstituted systems due to the presence of such a lag phase, which resulted in the misleading conclusion of apparent positive cooperativity (not shown).

Direct comparison of the V_{max} values calculated from the results shown above is not practical since we used SMPs as an enzyme source. However, we examined the ratio of the V_{max} values accompanying high K_m and low K_m values in the same assay. The ratio was found to be 11 (± 2) in the ATP hydrolytic reaction while it was 6 (± 2) in proton translocation. The reason for this discrepancy in the ratios has not yet been extensively elucidated, however, it may be due to the different H^+/ATP ratios for the reaction cycles.

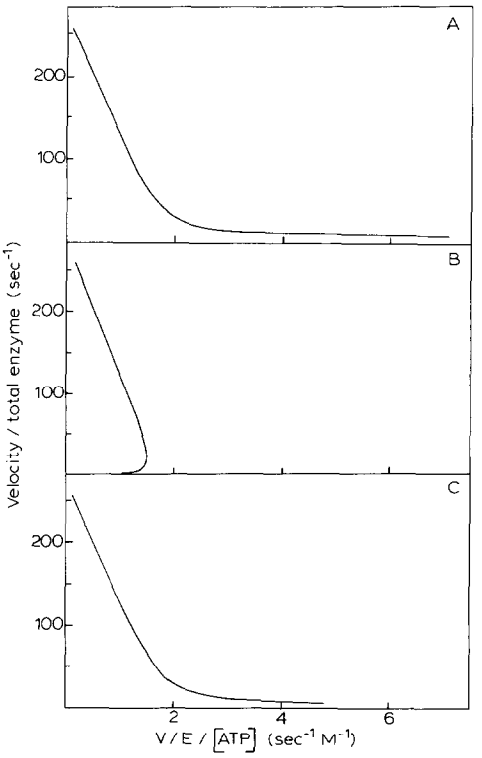

Fig.2. Simulated lines of Eadie Hofstee plots based on the alternate binding change model. (A) The total ATP hydrolysis reaction; (B) tri-site ATP hydrolysis reaction; (C) sum of bi-site and tri-site reaction. To convert the ordinate to units of (μmol·min^{-1}·mg^{-1}), multiply the values by $(60 \times 10^3)/(3.5 \times 10^5)$, and to convert the abscissa to units of (l·min^{-1}·mg^{-1}), multiply the values by $(60 \times 10^3)/(3.5 \times 10^5/1 \times 10^6)$.

3.2. *Proton translocation is coupled not only to the tri-site cycle*

We then examined the present data to the prediction based on the alternate binding change model of Boyer's group using the kinetic parameters and equations reported in [1]. Fig.2A is the theoretical curve of an Eadie Hofstee plot of the total ATPase activity over 1–2000 μM ATP which corresponds well to the results shown in fig.1A. Fig.2B is the theoretical curve simulated when only the tri-site catalysis is in progress. As mentioned above, if the scheme of the alternate binding model is correct, the kinetics of proton translocation should be in parallel with the tri-site ATPase activity and would reveal an apparent positive cooperativity as shown in fig.2B. However, this was clearly not the case (see fig.1B).

On the other hand, when the sum of bi-site and tri-site ATPase activities is plotted, an apparent negative cooperativity is revealed, as shown in fig.2C. Since the difference between fig.2A and C is very subtle and can only be observed at very low ATP concentration ranges (below 5 μM), it is not distinguishable from the present results whether proton translocation kinetics correspond to either fig.2A or C. However, it can be concluded that, at least, both bi-site and tri-site cycles participate in proton translocation even if the scheme of Boyer's group for the ATPase reaction is correct. Although the data are not shown, the shape of the Eadie Hofstee plot for proton translocation (fig.1B) could not be reconciled with the simulated curves of single site only, bi-site only, sum of single site and bi-site, or sum of single and tri-site catalysis.

3.3. *Other hypotheses*

In the present study, it was shown that both H^+ translocation and ATPase kinetics show negative cooperativity with the same K_m values. The results have been discussed in relation to the alternate binding change model with sequential participation of three catalytic sites as above. Now, other hypotheses assuming a different number of catalytic sites are to be discussed briefly in relation to proton translocation and the number of catalytic sites. If the number of catalytic site is two as proposed by Fellows et al. [10], the two K_m values may be attributed to each of the catalytic sites and both of them would be linked to proton translocation. The different nature of the two catalytic sites may be due to a regulatory site. If only one catalytic site is considered [11], a control site and/or a reaction scheme similar to that proposed for cytochrome oxidase [12] would be required in order to explain the concave nature of the Eadie Hofstee plot.

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