Steady-state kinetics of F_1-ATPase

Mechanism of anion activation

Oscar A. Roveri and Nora B. Calcaterra

Centro de Estudios Fotosintéticos y Bioquímicos (CONICET, Fund. M.Lillo and Universidad Nacional de Rosario), Suipacha 531, 2000 Rosario, Argentina

Received 22 August 1985

The kinetic behaviour of the ATPase activity of beef heart F_1 depends largely on the exposure of the enzyme to some anionic ligands such as sulphate and/or EDTA. F_1 prepared in the presence of such anions exhibited a triphasic kinetic pattern whereas F_1 from which those anions were removed by dialysis exhibited only two K_m values for ATP. Conversely to what has been previously reported, bicarbonate did not linearize F_1-ATPase kinetics. Moreover, anion activation cannot be simply explained by promotion of ADP release but mainly by an increase in affinity of the third catalytic site for ATP.

F_1-ATPase *Mitochondrial ATPase* *Anion activation* *Hysteretic enzyme* *Steady-state kinetics* *(Beef heart)*

1. INTRODUCTION

Multiple adenine nucleotide binding sites in the soluble mitochondrial F_1 have been suggested on the basis of kinetic studies. Ebel and Lardy [1], Recktenwald and Hess [2] and Gresser et al. [3] have reported biphasic steady-state kinetics (2 K_m values) for the ATPase activity of rat liver, yeast and beef heart F_1. Conversely, 3 K_m values have been reported for the rat liver and beef heart enzymes by Cerdán et al. [4] and Cross et al. [5]. Such a discrepancy remains unexplained as yet.

It has been reported [1,2] that F_1-ATPase kinetics are linearized by the presence of bicarbonate and other activating anions in the reaction media. Kasho and Boyer [6] have demonstrated that bicarbonate does not affect F_1 activity at low ATP concentrations, suggesting that in the presence of the anions biphasic rate behaviour also occurs. Bicarbonate activation at high ATP concentrations was postulated to be exerted by the promotion of ADP release.

Here, we show that the ATPase activity of beef heart F_1 exhibits triphasic kinetics in the absence and also in the presence of bicarbonate. We also show that the kinetic behaviour largely depends on the treatment of the enzyme before the experiments. The mechanism of activation by anions of the ATPase activity of F_1 is also discussed.

2. MATERIALS AND METHODS

Beef heart F_1 was prepared according to Knowles and Penefsky [7] and stored in liquid nitrogen in 250 mM sucrose, 2 mM EDTA, 4 mM ATP and 10 mM Tris-sulphate (pH 7.5). Before use, F_1 was desalted twice by the centrifuge column procedure described by Penefsky [8] using Sephadex (G-50 fine) equilibrated with 250 mM sucrose, 2 mM EDTA and 10 mM Tris-sulphate (pH 7.5).

Initial velocity assays were performed spectrophotometrically as described by Pullman et al. [9], at 30°C, in a reaction medium containing 100 mM sucrose, 1 mM $MgCl_2$, 4 mM phospho-*enol*pyruvate, 0.125 mM NADH, 60 IU pyruvate kinase, 50 IU lactate dehydrogenase and 40 mM Tris-HCl (pH 8). The reaction was started by

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

adding F_1 (3–5 μg protein) to the reaction media (2 ml). ATP was added as Mg-ATP, which was freshly prepared in a 1 : 1 Mg/ATP ratio by adding $MgCl_2$ to the ATP stock solution, in which ATP [10] and ADP [11] concentrations had been determined enzymatically.

Protein concentration was determined according to Lowry et al. [12] using bovine serum albumin as standard, the concentration of which had been determined spectrophotometrically (A_{279} = 6.67 cm^{-1} for 1% solution [13]).

3. RESULTS AND DISCUSSION

3.1. *Steady-state kinetics of F_1-ATPase: two or three K_m values for ATP?*

When the ATPase activity of the soluble beef heart mitochondrial ATPase (F_1) was measured at ATP concentrations ranging from 3 to 2000 μM, 3 clearly distinguishable phases could be observed (fig.1). Therefore, 3 different apparent K_m values could be estimated (K_m(I) = 4 μM, K_m(II) = 40 μM and K_m(III) = 500 μM), the corresponding V_{max} values being 2, 6 and 42 μmol/min per mg, respectively. These K_m values are quite similar to those reported by Cross et al. [5], except that our lowest K_m and V_{max} are several orders of magnitude higher than theirs. Such discrepancies can be due to differences in performing the enzymatic assays and/or in the enzyme preparations themselves.

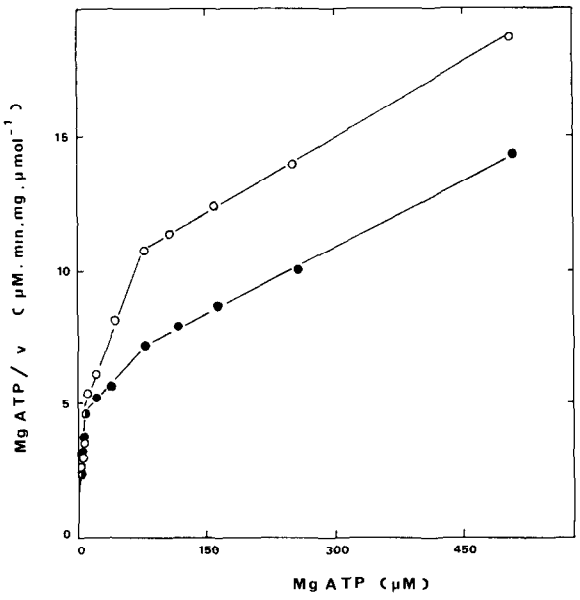

Fig.1. Hanes plot for the initial velocity of the ATPase activity of soluble beef heart F_1. Initial velocity measurements were carried out as indicated in section 2 in the absence (○) or presence (●) of 10 mM sodium bicarbonate. Values obtained at ATP concentrations from 500 to 2000 μM (not shown) belong to the same straight line that corresponds to the third phase.

Although our data can also be fitted to a reaction model with 2 catalytic sites and one regulatory site as proposed by Recktenwald and Hess [2] and Sloothaak et al. [14], here we are going to use the 3-catalytic-site model postulated by Gresser et al. [3] which has been more extensively described. Gresser et al. [3] have estimated the rate constants that made their 3-catalytic-site model (fig.2) consistent with their experimental results revealing 2 K_m values. To fit our results (fig.3) to that model, some of the rate constants reported by Gresser et al. [3] should be changed (see table 1). The more significant changes are that k_9 and k_8, in our case, are greater than k_5 and k_2 respectively, resulting in triphasic (fig.1) instead of biphasic kinetics [3]. Therefore, if the enzyme preparations used by different authors differ in the relative values of these rate constants, they would exhibit different kinetic behaviour (2 or 3 K_m values) even when they respond to a unique reaction scheme.

Such differences cannot be related to different experimental conditions used in the activity assay (i.e. free magnesium [4] or ATP concentrations), but rather to the enzyme preparation itself, since we have observed 3 K_m values under similar conditions to those used by others who reported 2 K_m values [1–3]. Since such discrepancies exist for F_1 prepared from the same source (cf. [3] with [5] and our results), it is possible that differences in purification or storing procedures or in treatment

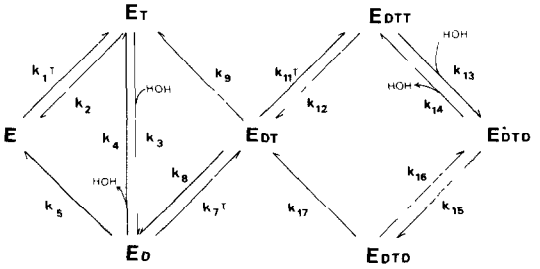

Fig.2. From [3] where T is ATP and D is ADP.

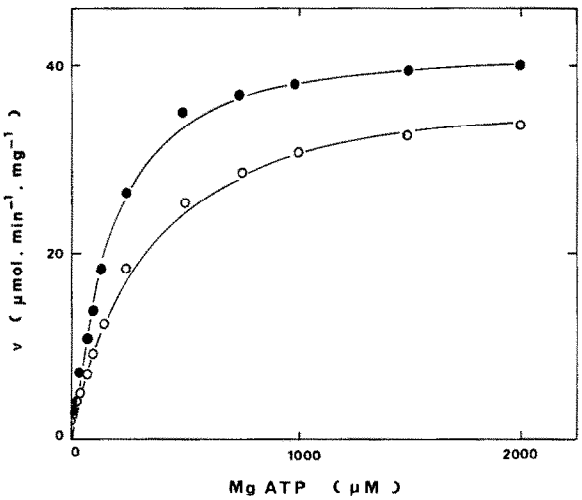

Fig.3. Steady-state kinetics of F_1-ATPase. The points indicate experimental measurements carried out in the absence (○) or presence (●) of 10 mM sodium bicarbonate. The lines give the predicted curves obtained according to the reaction scheme in [3], using the rate constant values shown in table 1.

of F_1 before the experiments can affect its kinetic behaviour. For instance, F_1 dialyzed against a buffer without sulphate and EDTA showed quite different rate behaviour from the non-dialyzed enzyme: V_{max} decreased by about 50% and the kinetics could simply be resolved (fig.4B) as the sum of 2 hyperbolas (2 K_m values) which was not possible for the enzyme before dialysis (fig.4A). This change in kinetic pattern is reversible, since V_{max} increases when the dialyzed enzyme is further dialyzed against a buffer containing sulphate and EDTA (not shown).

Therefore, the sole exposure of F_1 to some anionic ligands, e.g. sulphate and/or EDTA, which probably bind to slowly exchangeable sites [15], seems to alter its kinetic pattern. Di Pietro et al. [16] have reported an ADP-induced hysteretic inhibition of F_1. Although our results may be explained by hysteretic behaviour regarding anionic ligands, we do not believe that both phenomena are related, since our low-activity enzyme form (after dialysis) is still activated by bicarbonate (see

Table 1

Dissociation and rate constants for the F_1-ATPase

Constant	Values reported by Gresser et al. [3]	Values used in fig.3	
		Minus bicarbonate	Plus bicarbonate
k_1	$6.0 \times 10^7\ M^{-1} \cdot s^{-1}$	id[a]	id[b]
k_2	$8.5 \times 10^1\ s^{-1}$	$4.0 \times 10^2\ s^{-1}$	id
K_d(I)	1.4×10^{-6} M	6.7×10^{-6} M	id
k_3	$5.0 \times 10^2\ s^{-1}$	$1.0 \times 10^3\ s^{-1}$	id
k_4	$5.0 \times 10^2\ s^{-1}$	$5.5 \times 10^2\ s^{-1}$	id
k_5	$9.6\ s^{-1}$	$1.8 \times 10^1\ s^{-1}$	id
k_7	$9.0 \times 10^7\ M^{-1} \cdot s^{-1}$	id	id
k_8	$4.0 \times 10^1\ s^{-1}$	$2.8 \times 10^3\ s^{-1}$	id
K_d(II)	4.4×10^{-7} M	3.1×10^{-5} M	id
k_9	$8.5\ s^{-1}$	$2.8 \times 10^1\ s^{-1}$	id
k_{11}	$1.8 \times 10^6\ M^{-1} \cdot s^{-1}$	id	id
k_{12}	$1.0 \times 10^2\ s^{-1}$	$1.9 \times 10^3\ s^{-1}$	$1.0 \times 10^2\ s^{-1}$
K_d(III)	$5.5 \times 10^{-5}\ M^{-1} \cdot s^{-1}$	1.0×10^{-3} M	5.5×10^{-5} M
k_{13}	$1.0 \times 10^4\ s^{-1}$	$1.0 \times 10^3\ s^{-1}$	id
k_{14}	$1.0 \times 10^4\ s^{-1}$	$1.0 \times 10^3\ s^{-1}$	id
k_{15}	$1.0 \times 10^7\ s^{-1}$	id	id
k_{16}	$3.0 \times 10^2\ s^{-1}$	id	id
k_{17}	$2.9 \times 10^2\ s^{-1}$	$3.1 \times 10^2\ s^{-1}$	$3.4 \times 10^2\ s^{-1}$

[a] Values equal to that reported by Gresser et al. [3] (second column)
[b] Similar values to those shown in the second column (minus bicarbonate)

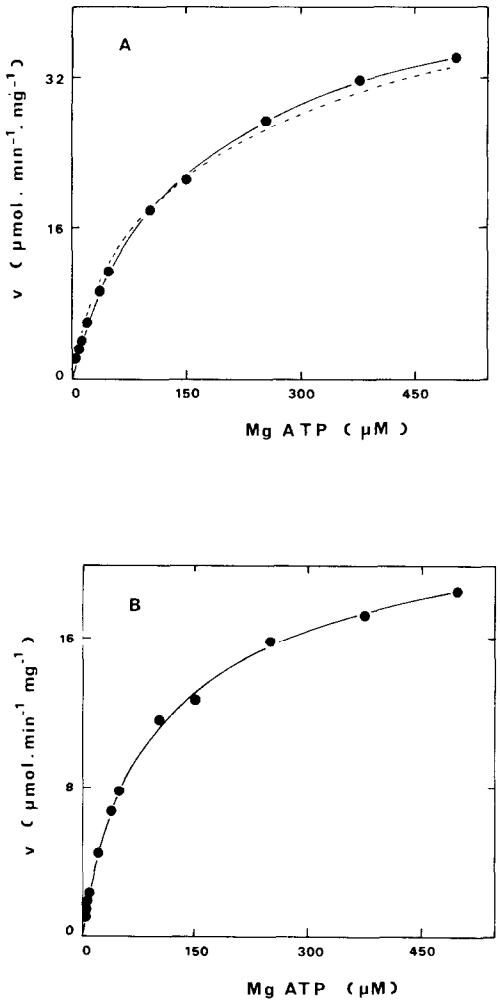

Fig.4. Dependence of the kinetic pattern of the F_1-ATPase on the treatment of the enzyme. F_1 was desalted as indicated in section 2 and, when indicated, dialyzed for 2 h against 250 mM sucrose, 10 mM Tris-HCl (pH 7.5) and 10 mM NaCl, at room temperature with 3 changes of dialysis buffer. Initial velocity measurements were performed with the non-dialyzed (A) and dialyzed (B) enzymes. (A) The dashed line was obtained by fitting the experimental points to the sum of 2 hyperbolas, using the computer program 2SYTEHYP from Barlow [17], which is based on a least-squares fitting procedure (K_m = 12 and 230 μM and V_{max} = 7 and 39 μmol/min per mg). The solid line represents the fit to the sum of 3 hyperbolas (K_m = 5, 40 and 250 μM; V_{max} = 2, 7 and 36 μmol/min per mg). (B) The solid line was obtained as indicated for the dashed one in (A) (K_m = 10 and 145 μM; V_{max} = 4 and 20 μmol/min per mg).

section 3.2), whereas F_1 inhibited by ADP binding was reported to be insensitive to HCO_3^- [17].

3.2. *Anion activation of the ATPase activity of soluble F_1*

Also in the presence of 10 mM bicarbonate, 3 clearly distinguishable phases were observed (fig.1). Although this result does not agree with those of Ebel and Lardy [1] and Recktenwald and Hess [2] who reported only one K_m in the presence of activating anions, it must be noted that in our experiments (fig.1) we expanded the range of low ATP concentrations used by them.

From the Hanes plot in fig.1, 3 K_m values could be estimated in the presence of bicarbonate: K_m(I) = 4 μM, K_m(II) = 100 μM and K_m(III) = 350 μM. The corresponding V_{max} values were: 2.5, 11.5 and 53.5 μmol/min per mg. When these values are compared with those obtained in the absence of bicarbonate (see section 3.1), it can be clearly seen that bicarbonate hardly affects the initial velocity of ATP hydrolysis at low ATP concentrations (K_m(I) region). K_m(II) is increased and K_m(III) is decreased by bicarbonate. The V_{max} values are increased by the anion, especially that corresponding to K_m(II) which is increased by 100%. Similar results were obtained when F_1 dialyzed as indicated in section 3.1 was used (not shown).

Other activating anions, such as sulphite and dinitrophenolate, behave similarly to bicarbonate (not shown).

The anions tested do not affect the enzyme activity when the enzyme is operating as a single-site enzyme, viz. at low ATP concentrations; therefore, 2 catalytic sites must be operative to allow bicarbonate to exert its action. The action of bicarbonate at higher ATP concentrations cannot be simply explained by an acceleration of ADP release, as postulated by Kasho and Boyer [6], since for fitting to the model of Boyer et al. [3] our results obtained in the presence of bicarbonate, it is not sufficient to increase only the values of the rate constants that govern ADP release (k_9 and k_{17}) at high ATP, but also k_{12} (k_{off} of ATP from the third site) (see table 1 and fig.3). Actually, a more dramatic change must be produced in k_{12} than in k_{17}. Assuming that the model of Boyer et al. [3] is valid, the most simple hypothesis to explain the action of bicarbonate is that the anion diminishes the

differences between the $K_{d(ATP)}$ for the second and third catalytic sites, also promoting the departure of ADP from the third site.

In summary, activating anions modify the interaction between the second and third catalytic sites, strongly attenuating the negative cooperativity for the binding of ATP (see table 1) and slightly favouring the positive cooperativity for catalysis.

The present results clearly indicate that the same enzyme preparation can yield, in the range 3–2000 μM, either 2 or 3 K_m values, depending on the exposure of the enzyme to some common anionic ligands such as sulphate and EDTA. Therefore, to compare results obtained in different laboratories, it is extremely important to establish not only how the enzyme is prepared and stored, but also how the enzyme is treated before the experiments.

ACKNOWLEDGEMENTS

O.A.R. is a member of the Carrera del Investigador from the Consejo Nacional de Investigaciones Científicas y Técnicas from the República Argentina and N.B.C. is a fellow from the same Institution. This work was supported by grants from CONICET.

REFERENCES

[1] Ebel, R.E. and Lardy, H.A. (1975) J. Biol. Chem. 250, 191–196.
[2] Recktenwald, D. and Hess, B. (1977) FEBS Lett. 76, 25–28.
[3] Gresser, M.J., Myers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038.
[4] Cerdán, E., Campo, M.L., López-Moratalla, N. and Santiago, E. (1983) FEBS Lett. 158, 151–153.
[5] Cross, R.L., Grubmeyer, C. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12101–12105.
[6] Kasho, V.N. and Boyer, P.D. (1984) J. Bioenerg. Biomembranes 16, 407–419.
[7] Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617–6623.
[8] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[9] Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329.
[10] Lamprecht, D. and Trautschold, I. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H.-U. ed.) vol.4, pp.2101–2110, 2nd Engl. edn, Academic Press, New York.
[11] Jaworek, D., Gruber, H. and Bergmeyer, H.-U. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H.-U. ed.) vol.4, pp.2127–2129, 2nd Engl. edn, Academic Press, New York.
[12] Lowry, O.H., Rosebrough, A.L., Farr, L.H. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Foster, J.F. and Sterman, M.D. (1956) J. Am. Chem. Soc. 78, 3656–3660.
[14] Sloothaak, J.B., Berden, J.A., Herweijer, M.A. and Kemp, A. (1985) Biochim. Biophys. Acta 809, 27–38.
[15] Recktenwald, D. and Hess, B. (1980) Biochim. Biophys. Acta 592, 377–384.
[16] Di Pietro, A., Penin, F., Godinot, C. and Gautheron, D. (1980) Biochemistry 19, 5671–5678.
[17] Baubichon, H., Di Pietro, A., Godinot, C. and Gautheron, D. (1982) FEBS Lett. 137, 261–264.
[18] Barlow, R.B. (1983) in: Biodata Handling with Microcomputers, pp.172–175, Elsevier, Amsterdam, New York.