

Mitochondrial adenosine triphosphatase from human placenta — inhibition by free magnesium ions of ITP hydrolysis*

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The effects of Mg^{2+} and bicarbonate on the kinetics of ITP hydrolysis by soluble ATPase (F_1) from human placental mitochondria were studied. Increasing amounts of Mg^{2+} at fixed ITP concentration, caused a marked activation of F_1 followed by inhibition at higher Mg^{2+} concentration. The appropriate substrate for the mitochondrial F_1 seems to be the $MgITP$ complex as almost no ITP was hydrolysed in the absence of magnesium.

Mg^{2+} behaved as a competitive inhibitor towards the $MgITP$ complex. In this respect the human placental enzyme differ from that from other sources such as yeast, beef liver or rat liver. The linearity of the plot presenting competitive inhibition by free Mg^{2+} of $MgITP$ hydrolysis (in the presence of activating bicarbonate anion) suggests that both Mg^{2+} and $MgITP$ bind to the same catalytic site ($K_m(MgITP) = 0.46$ mM, $K_i(Mg) = 4$ mM).

When bicarbonate was absent in the ITPase assay, placental F_1 exhibited apparent negative cooperativity in the presence of 5 mM Mg^{2+} , just as it did with $MgATP$ as a substrate under similar conditions. Bicarbonate ions eliminated the negative cooperativity with respect to ITP (as the Hill coefficient of 0.46 was brought to approx. 1), and thus limited inhibition by free Mg^{2+} .

The results presented suggest that the concentration of free magnesium ions may be an important regulatory factor of the human placental F_1 activity.

The mitochondrial coupling factor 1 (F_1) is a catalytic component of the membrane-bound proton-translocating ATP synthase complex (F_1F_0) (EC 3.6.1.34) which catalyzes *in vivo* ATP synthesis in the course of oxidative phosphorylation. Solubilized F_1 catalyzes *in vitro* only hydrolysis of nucleotides (ATP, GTP, ITP). It is generally accepted that F_1 is composed of five

types of subunits, referred to as α , β , γ , δ and ϵ . In all the species examined to date, mitochondrial F_1 contains three copies of α - and three copies of β -subunits, together with single copies of γ -, δ - and ϵ -subunits [1-3]. Catalytic sites are located on the β -subunits or at interfaces between α - and β -subunits. Catalytic properties of the mitochondrial F_1 suggest that

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¹ Abbreviations: ATP, adenosine triphosphate; F_1 , the coupling factor 1, (soluble ATPase); ITP, inosine triphosphate; Mg , free magnesium ions.

Enzymes: ATP synthase, proton translocating ATP synthase (EC 3.6.1.34); lactate dehydrogenase, L-lactate:NAD⁺ oxidoreductase (EC 1.1.1.27); pyruvate kinase, ATP:pyruvate 2-O-phosphotransferase (EC 2.7.1.40).

hydrolysis of ATP by this enzyme involves cooperative interactions between nucleotide-binding sites [4–7]. Negative cooperativity between substrate sites expressed by a Hill coefficient below 1 is abolished in the presence of appropriate anions, like bicarbonate. Boyer and co-workers [8] proposed that the synthesis of F₁-bound ATP from F₁-bound ADP and P_i does not require energy, and that energy input into mitochondrial system was needed for the release of ATP from the enzyme. This release of the reaction product from one catalytic site was facilitated by simultaneous binding of the substrates in a second, interacting site. This binding change mechanism (for reviews see [9–11]), is considered a model for explaining the mechanism of ATP synthases.

Magnesium ions play an essential role in enzymatic hydrolysis of ATP and other nucleotides. It is generally recognized that MgATP is the true substrate for mitochondrial F₁-ATPase [12]. This conclusion has been drawn among others from the fact that free magnesium ions could act as a competitive inhibitor towards MgATP [13–16]. Wakagi & Ohta [16] suggested that the inhibitory effect of free Mg²⁺ is fully specific for ATP as a substrate.

In a previous paper from this laboratory [15] it has been shown that the degree of deviation from the Michaelis-Menten kinetics for MgATP hydrolysis (in Tris/HCl buffer) depends on free Mg²⁺ ion concentration; the Hill coefficient of 0.75 at 1 mM Mg²⁺ was brought to approx. 0.5 at 5 mM concentration of free Mg²⁺. The purpose of this paper was to study effect of Mg²⁺ and bicarbonate on MgITP hydrolysis catalyzed by F₁ purified in this laboratory from human placenta. The results reported previously [15] and here indicate that hydrolysis of nucleotides (not only ATP) by placenta mitochondrial F₁ involves cooperative interactions between catalytic sites.

MATERIALS AND METHODS

ITP, NADH, phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase solution were obtained from Sigma Chemical Co. as crystalline suspensions in (NH₄)₂SO₄. All other chemicals were of reagent grade. The substrate purification procedure and its purity assay were performed as describe previously [15].

Human placenta mitochondrial F₁ was isolated by chloroform extraction and purified by ion-exchange chromatography [15]. Shortly prior to use, an aliquot of the F₁ suspension was centrifuged to remove (NH₄)₂SO₄ and ATP which were present in the storage medium, and the sediment was redissolved in 50 mM Tris/HCl buffer, pH 8.0, containing 30% glycerol. Specific activity of the glycerol-stabilized F₁ solution was increased by about 30% in the first 2 h and then remained fairly constant during the next 48 h at room temperature. The protein content of F₁ solution was determined by the Coomassie blue G-250 dye-binding method [17], using crystalline bovine serum albumin as a standard.

ITPase activity of mitochondrial F₁ was measured in the presence or absence of the ITP-regenerating system at 37°C, in the total volume of 1 ml, by the spectrophotometric method [18] with slight modifications. The nonspecificity of pyruvate kinase for nucleotide diphosphates has been described by Kayne [19]. In the assay run in the presence of ITP-regenerating system the reaction mixture consisted of 50 mM Tris/HCl, pH 8.1, 2 mM potassium phosphoenolpyruvate, 0.3 mM NADH, 24 units of pyruvate kinase, 15 units of lactate dehydrogenase, 1 or 5 mM magnesium ions as MgCl₂, and Mg-ITP complex as indicated in the legends to Figures. The reaction was started by addition of the enzyme solution (10 µl) to the assay mixture and the change in absorbance at 340 nm was followed in a recording double beam spectrophotometer equipped with temperature control. F₁ concentration varied between 1.2 and 2.0 µg protein/ml.

Alternatively, ITPase activity was measured in the experiments with constant ITP concentration and increasing magnesium concentrations as indicated in Fig. 2. The assay mixture contained 50 mM Tris/HCl, pH 8.1, 30 mM KCl and 0.85 mM ITP (Na salt). After 3 min preincubation at 37°C, the reaction was started by adding the enzyme; the final volume was 1 ml. After 3 min the reaction was terminated by addition of 50 µl of 60% perchloric acid. Then 0.5 ml aliquots of the neutralized perchloric acid extracts were analyzed for IDP content. IDP measurement was performed in the total volume of 1 ml of mixture containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 20 units of

pyruvate kinase and 20 units of lactate hydrogenase.

The apparent stability constant for MgITP was calculated using the empirical formula as described by Adolfsen & Moudrianakis [20].

RESULTS AND DISCUSSION

Several experiments with mitochondrial F₁ [4, 21, 22] indicate that dependence of ITP hydrolysis rate on substrate concentration obeys Michaelis-Menten kinetics, in contrast to the complex non-Michaelis kinetics of ATP hydrolysis by F₁ under standard conditions. Previous experiments in this laboratory [15] showed that the apparent negative cooperativity of F₁-ATPase activity is dependent on concentration of free Mg²⁺ ions. If the hydrolysis of nucleotides by F₁ involved cooperative interactions between catalytic sites, one would expect that, at high concentration of free Mg²⁺ ions, the kinetics of ITP hydrolysis would be "biphasic". In the experiment shown in Fig. 1, ITPase activity of the placenta mitochondrial F₁ was measured at varying concentrations of MgITP with 5 mM

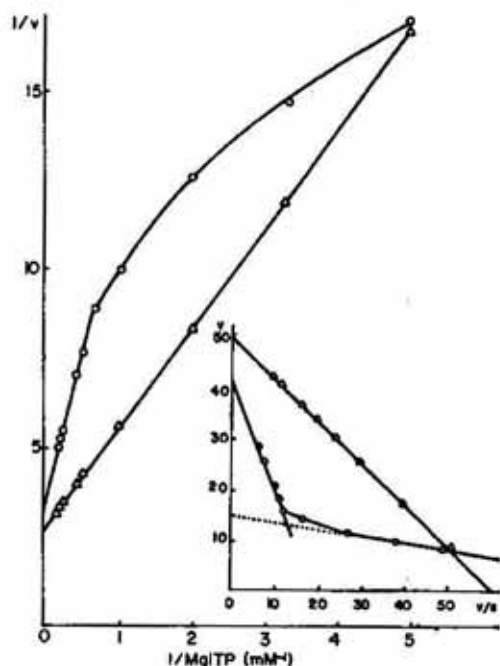


Fig. 1. ITPase activity of placenta mitochondrial F₁ as a function of MgITP concentration in the presence (Δ) or absence (\circ) of 30 mM bicarbonate. Spectrophotometric assays were carried out as described under Materials and Methods. The incubation medium contained 5 mM free Mg²⁺. The insert shows the Eadie-Hofstee plot.

excess of Mg²⁺ ions either in the presence or absence of bicarbonate at pH 8.1. Lineweaver-Burk plots of the kinetic data for MgITP hydrolysis in the absence of bicarbonate deviated considerably from the typical Michaelis-Menten function. Eadie-Hofstee plot was used to obtain kinetic constants (Fig. 1, insert). This plot is biphasic with two distinct slopes. Over the substrate concentration range of 0.2–1 mM, hydrolysis of MgITP occurred with an apparent K_m equal to 0.16 mM. The hydrolysis of the substrate at higher concentrations occurred with an apparent K_m equal to 2.7 mM. Previously, Wong *et al.* [23] studying the kinetics of MgITP hydrolysis by bovine heart mitochondrial ATPase, observed also two K_m values in the absence of KHCO₃. As can be seen in Fig. 1, placental F₁ exhibited apparent negative cooperativity with MgITP as the substrate in Tris/HCl medium (just as it did with MgATP under similar conditions). The Hill coefficient in the presence of 5 mM free Mg²⁺ ion was found to be about 0.5, while in the presence of 1 mM free Mg²⁺ ion it was, 1. Higher concentrations of bicarbonate eliminated the negative cooperativity (Fig. 1). At 30 mM KHCO₃ concentration linear plots were observed, the maximum velocity increased about 1.2-fold and the Hill coefficient of 0.46 was brought to approx. 1. Over the substrate concentration range of 0.2–5 mM, hydrolysis of ITP occurred with an apparent K_m , equal to 1.08 mM. Previously, a similar effect of bicarbonate on ATP hydrolysis has been reported [4, 15]. The results reported previously [15] and here show no differences between ATP and ITP as far as the kinetics of hydrolysis of those two compounds in the presence of 5 mM Mg²⁺ ions is concerned. The results presented suggest that hydrolysis of nucleotides (not only ATP) by placenta mitochondrial F₁ involves cooperative interactions between catalytic sites.

Several workers have reported that free magnesium might act as a competitive inhibitor towards MgATP in ATP hydrolysis catalyzed by mitochondrial F₁ [13, 15]. According to Wakagi & Ohta [16], the inhibitory action of Mg²⁺ was fully specific for ATP as a substrate, as an excess of Mg²⁺ had no effect on GTP or ITP hydrolysis by beef liver mitochondrial F₁. Data illustrating the influence of Mg²⁺ concentration on ITP hydrolysis by placental F₁ are shown in Fig. 2, in which the release of IDP is plotted against MgCl₂ concentration. In the absence of

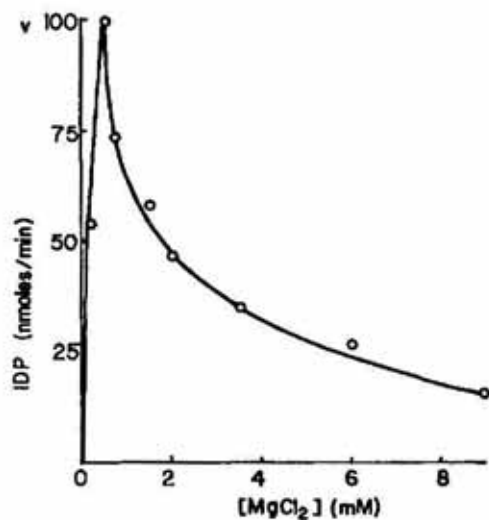


Fig. 2. ITPase activity of placenta mitochondrial F_1 at a constant added ITP concentration (0.85 mM) and increasing $MgCl_2$ concentrations. Activity was measured as described under Materials and Methods.

$MgCl_2$ ITP was not hydrolysed. As the concentration of added $MgCl_2$ increased, the ITPase activity of mitochondrial F_1 increased up to a maximum and then decreased. The highest velocity was observed at $MgCl_2$ concentration slightly lower than that of ITP. This optimum indicates that Mg^{2+} is an apparent inhibitor at

high concentrations. This feature is different from the results obtained for ITP hydrolysis catalyzed by yeast F_1 [24], beef liver F_1 [16] or rat liver F_1 (Aleksandrowicz, unpublished), which was not affected by Mg^{2+} given in excess.

To elucidate the mode of action of free Mg^{2+} ions on ITPase activity of mitochondrial F_1 , some kinetic parameters were examined. For the placental mitochondrial F_1 , free Mg^{2+} ion was a competitive inhibitor towards Mg ITP in the presence of bicarbonate (Fig. 3A) as it modified the affinity of the enzyme for its substrate only. Free Mg^{2+} ions did not affect the Hill coefficient of ITPase activity (1.0 in all cases) under the conditions applied; F_1 exhibited a Michaelian behaviour in the presence of Mg^{2+} in excess. The replot of slopes of the straight lines obtained in Fig. 3A and the plot of apparent K_m in the presence of inhibitor versus Mg^{2+} ions are linear (Fig. 3B and C). This might indicate that only one Mg^{2+} ion per catalytic site of the enzyme is required to induce the inhibition. The "control" K_m value (in the absence of free Mg^{2+}) for Mg ITP can be graphically extrapolated either from Fig. 3A or from Fig. 3C; in both cases the value of 0.46 mM was obtained. Dixon plots for different fixed Mg ITP concentrations (Fig. 4) were linear and gave a K_i value of 4 mM

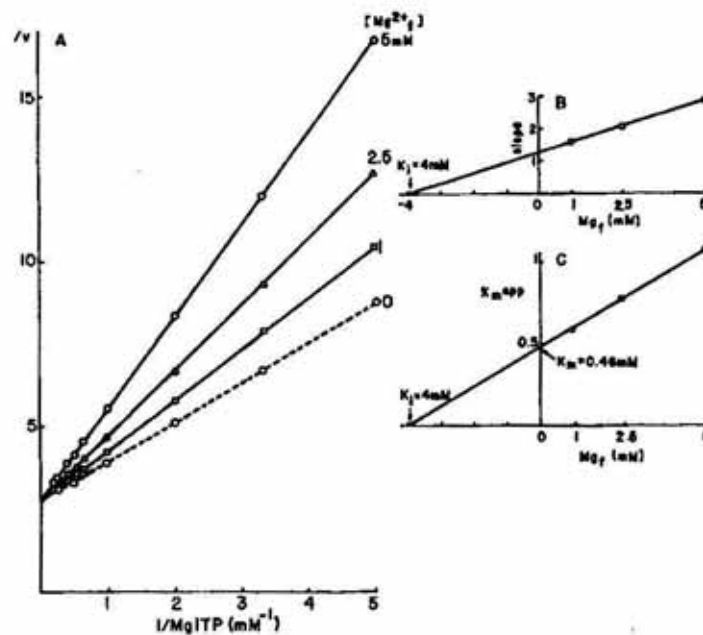


Fig. 3. Inhibition of ITPase activity of placenta mitochondrial F_1 by free Mg^{2+} in the presence of 30 mM bicarbonate.

The points (○) of the dotted line were graphically determined from Fig. 4 after extrapolation to free $Mg^{2+} = 0$. A. Double reciprocal plot. B. Slope replot. C. Secondary plot of K_m versus free Mg^{2+} concentration.

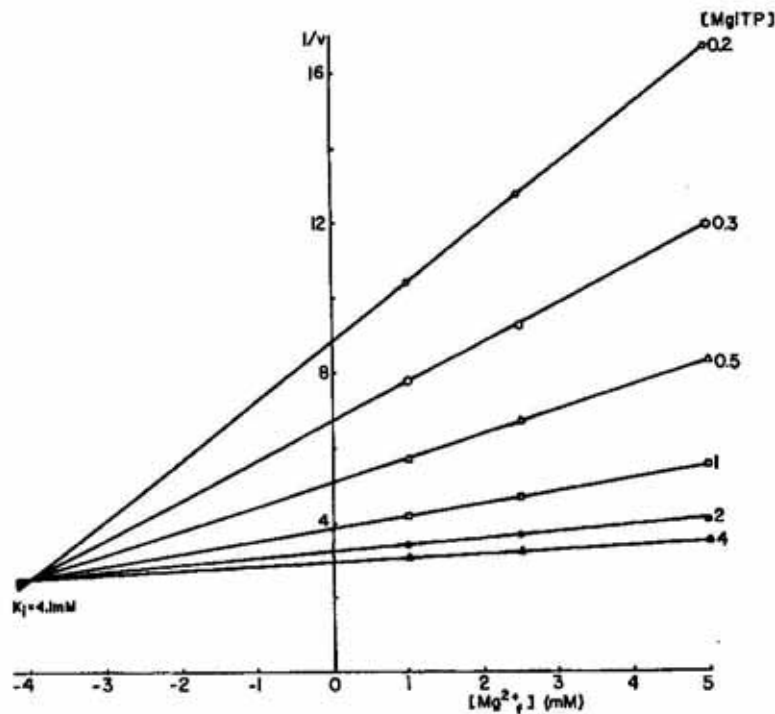


Fig. 4. Dixon plot to determine K_i for free Mg^{2+} acting as a competitive inhibitor of the hydrolysis of $MgITP$ by placenta mitochondrial F_1 .

The $1/v$ values from Fig. 3A were extrapolated to constant $MgITP$ concentrations. $MgITP$ was used at the indicated millimolar concentrations.

for free Mg^{2+} . The v value for different ITP concentrations in the absence of free Mg^{2+} extrapolated from these plots allowed to calculate the Hill coefficient of 1.0 for $MgITP$. In the absence of bicarbonate, free Mg^{2+} was again a competitive inhibitor of the placental F_1 (Fig. 5).

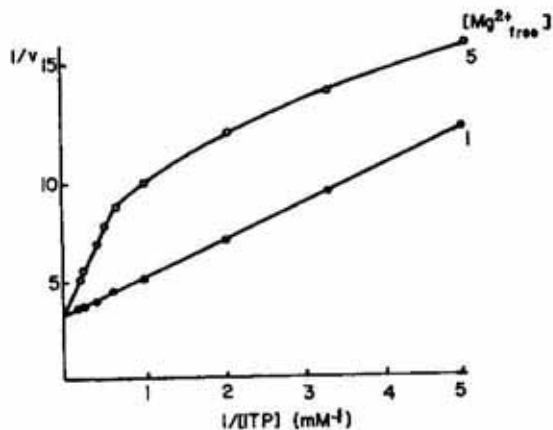


Fig. 5. Inhibition of $ITPase$ activity of placenta mitochondrial F_1 by free Mg^{2+} in the absence of bicarbonate.

Spectrophotometric assays were carried out as described under Materials and Methods. Free Mg^{2+} was used at the indicated millimolar concentrations.

It should be noted that inhibition by Mg^{2+} is not simply competitive, as negative cooperativity between catalytic sites was induced. This indicates the existence of at least two binding sites for the substrate. It is possible that interactions between the sites are promoted by a conformational change induced by free Mg^{2+} ions. The data presented demonstrate that a negative cooperativity between $MgITP$ sites exists (in the absence of an activating anion) provided free Mg^{2+} is present in excess.

Finally, the results presented in this study suggest: i) that both Mg^{2+} , as well as $MgITP$, bind to the catalytic site of placental F_1 ; ii) that hydrolysis of both ATP and ITP by F_1 involves cooperative interactions between catalytic sites, and iii) that concentration of free Mg^{2+} ions may be an important regulator of the enzyme activity. The results reported previously [15, 25] and here indicate that cooperative interaction between catalytic sites on separate subunit may be a fundamental feature of the mechanism of the human placental F_1 - $ATPase$ action.

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REFERENCES

1. Senior, A.E. (1988) ATP synthesis by oxidative phosphorylation. *Physiol. Rev.* **68**, 177–231.
2. Penefsky, H.S. & Cross, R.L. (1991) Structure and mechanism of FoF₁-type ATP synthases and ATPases; in: *Advances in Enzymology and Related Areas of Molecular Biology* (Meister, A., ed.) vol. 64, pp. 173–214, John Wiley & Sons, Inc., New York.
3. Issartel, J.P., Dupuis, A., Garin, J., Lunardi, J., Michel, L. & Vignais, P.V. (1992) The ATP synthase (F₀-F₁) complex in oxidative phosphorylation. *Experientia* **48**, 351–362.
4. Schuster, S.M., Ebel, R.E. & Lardy, H.A. (1975) Kinetic studies on rat liver and beef heart mitochondrial ATPase. Evidence for nucleotide binding at separate regulatory and catalytic sites. *J. Biol. Chem.* **250**, 7848–7853.
5. Grubmeyer, C. & Penefsky, H.S. (1981) The presence of two hydrolytic sites on beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **256**, 3718–3727.
6. Cross, R.L., Grubmeyer, C. & Penefsky, H.S. (1982) Mechanism of ATP hydrolysis by beef heart mitochondrial ATPases. Rate enhancements resulting from cooperative interactions between multiple catalytic sites. *J. Biol. Chem.* **257**, 12101–12105.
7. Berden, J.A., Hartog, A.F. & Edel, C.M. (1991) Hydrolysis of ATP by F₁ can be described only on the basis of dual-site mechanism. *Biochim. Biophys. Acta* **1057**, 151–156.
8. Boyer, P.D., Cross, R.L. & Momsen, W. (1973) A new concept for energy coupling in oxidative phosphorylation based on a molecular explanation of the oxygen exchange reactions. *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2837–2839.
9. Boyer, P.D. (1987) The unusual enzymology of ATP synthase. *Biochemistry* **26**, 8503–8507.
10. Boyer, P.D. (1989) A perspective of the binding change mechanism for ATP synthesis. *FASEB J.* **3**, 2164–2178.
11. Cross, R.L. (1988) The number of functional catalytic sites on F₁-ATPases and the effects of quarternary structural asymmetry on their properties. *J. Bioenerg. Biomembr.* **20**, 395–405.
12. Gruys, K.J., Urbauer, J.L. & Schuster, S.M. (1985) Metal-nucleotide structural characteristics during catalysis by beef heart mitochondrial F₁. *J. Biol. Chem.* **260**, 6533–6540.
13. Selwyn, M.J. (1967) Preparation and general properties of a soluble adenosine triphosphatase from mitochondria. *Biochem. J.* **105**, 279–288.
14. Fleury, B., Di Petro, A., Godinot, C. & Gautheron, D.C. (1980) Role of magnesium on kinetic parameters of soluble F₁-ATPase from pig heart mitochondria. *Biochimie* **62**, 733–737.
15. Aleksandrowicz, Z. (1980) Mitochondrial adenosine triphosphatase from human placenta — Purification and catalytic properties. *Int. J. Biochem.* **11**, 165–175.
16. Wakagi, T. & Ohta, T. (1981) Implications of the existence of two states of beef liver mitochondrial adenosine triphosphatase as revealed by kinetic studies. *J. Biochem. (Tokyo)* **89**, 1205–1213.
17. Spector, T. (1978) Refinement of the Coomassie blue method of protein quantitation. *Anal. Biochem.* **86**, 142–146.
18. Ebel, R.E. & Lardy, H.A. (1975) Stimulation of rat liver mitochondrial adenosine triphosphatase by anions. *J. Biol. Chem.* **250**, 191–196.
19. Kayne, F.J. (1973) Pyruvate kinase; in: *The Enzymes* (Boyer, P.D., ed.) vol. 8, pp. 353–382, Academic Press, New York.
20. Adolfsen, R. & Moudrianakis, E.N. (1978) Control of complex metal ion equilibria in biochemical reaction systems. Intrinsic and apparent stability constants of metal-adenine nucleotide complexes. *J. Biol. Chem.* **253**, 4378–4379.
21. Pedersen, P.L. (1976) ATP-dependent reactions catalyzed by inner membrane vesicles of rat liver mitochondria. Kinetics, substrate specificity, and bicarbonate sensitivity. *J. Biol. Chem.* **251**, 934–940.
22. Takeshige, K., Hess, B., Bohm, M. & Zimmerman-Telschow, H. (1976) Mitochondrial adenosine triphosphatase from yeast, *Saccharomyces cerevisiae*. Purification, subunit structure and kinetics. *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1605–1622.
23. Wong, S.-Y., Matsuno-Yagi, A. & Hatefi, Y. (1984) Kinetics of ATP hydrolysis by F₁-ATPase and the effects of anion activation, removal of tightly bound nucleotides, and partial inhibition of the ATPase by covalent modification. *Biochemistry* **23**, 5004–5009.
24. Rectenwald, D. & Hess, B. (1980) Classification of nucleotide binding sites on mitochondrial F₁-ATPase from yeast. *Biochim. Biophys. Acta* **592**, 377–384.
25. Aleksandrowicz, Z. (1985) Mitochondrial adenosine triphosphatase from human placenta. — Effects of adenylyl and guanylyl imidodiphosphate. *Int. J. Biochem.* **17**, 229–234.