Biochemistry (Moscow), Vol. 64, No. 10, 1999, pp. 1128-1137. Translated from Biokhimiya, Vol. 64, No. 10, 1999, pp. 1337-1347.

Kinetic Mechanism of $F_o \cdot F_1$ Mitochondrial ATPase: Mg^{2+} Requirement for Mg·ATP Hydrolysis

A. V. Syroeshkin, M. A. Galkin, A. V. Sedlov, and A. D. Vinogradov*

Department of Biochemistry, School of Biology, Lomonosov Moscow State University, Moscow, 119899 Russia; fax: (7-095) 939-3955; E-mail: adv@biochem.bio.msu.su

Received February 4, 1999
Revision received March 9, 1999

Abstract—The initial rates of ATP hydrolysis catalyzed by $F_o \cdot F_1$ (bovine heart submitochondrial particles) preincubated in the presence of P_i for complete activation of the oligomycin-sensitive ATPase were measured as a function of ATP, Mg^{2+}, and Mg·ATP concentrations. The results suggest the mechanism in which Mg·ATP complex is the true substrate of the ATPase and the second Mg^{2+} bound at a specific pH-dependent site is needed for the catalysis. Simple hyperbolic Michaelis–Menten dependences of the reaction rate on the substrate (Mg·ATP) and activating Mg^{2+} were found. In contrast to the generally accepted view, no inhibition of ATPase by free Mg^{2+} was found. Inhibition of the reaction by free ATP is due to a decrease of free Mg^{2+} needed for the catalysis. In the presence of both Ca^{2+} and Mg^{2+} the kinetics of ATP hydrolysis suggest that the Ca·ATP complex is neither hydrolyzed nor competes with Mg·ATP, and free Ca^{2+} does not affect the hydrolysis of Mg·ATP complex. A crucial role of free Mg^{2+} in the time-dependent inhibition of ATPase by azide is shown. The dependence of apparent K_m for Mg·ATP on saturation of the Mg^{2+}-specific site suggests the formal ping-pong mechanism in which bound Mg^{2+} participates in the overall reaction after dissociation of one product (most likely P_i) thus promoting either release of ADP (catalytic turnover) or slow isomerization of the enzyme–product complex (formation of the dead-end ADP(Mg^{2+})-inhibited enzyme). The rate of Mg·ATP hydrolysis only slightly depends on pH at saturating Mg^{2+}. In the presence of limited amounts of free Mg^{2+} the pH dependence of the initial rate corresponds to the titration of a single group with $pK_a = 7.5$. The simple competition between H^+ and activating Mg^{2+} was observed. The specific role of Mg^{2+} as a coupling cation for energy transduction in $F_o \cdot F_1$-ATPase is discussed.

Key words: $F_o \cdot F_1$-ATP synthase, F_1-ATPase, mitochondria, ATP hydrolysis, oxidative phosphorylation

Oxidative phosphorylation and photophosphorylation are catalyzed by $F_o \cdot F_1$-ATP synthases which are oligomeric proteins embedded in the coupling membranes of mitochondria, chloroplasts, and bacteria. The F_1 component (a peripheral part of ATP synthase) has molecular mass of about 380 kD and is composed of five different polypeptide chains in the stoichiometry $\alpha_3 \cdot \beta_3 \cdot \gamma \cdot \delta \cdot \varepsilon$ [1-3]. F_1 units from various sources, being energetically uncoupled, rapidly hydrolyze ATP in the presence of bivalent cations, and their catalytic properties have been extensively examined for more than 35 years since the enzyme was first isolated [4]. The 2.8 Å resolution structure for bovine mitochondrial F_1, which is the largest asymmetric protein structure ever solved, recently become available [5].

The membrane-embedded hydrophobic F_o part contains at least three types of subunits (*a*, *b*, and *c* in stoichiometry $a \cdot b_2 \cdot c_{9\text{-}12}$ in *E. coli* [6]) and has more complex structure for bovine heart F_o [7]. The F_o complex contains a proton pathway conducting protons coupled by an unknown mechanism with ATP synthesis at the F_1 active site(s) at the expense of an electrochemical H^+ gradient ($\Delta\bar{\mu}_{H^+}$) across the coupling membrane. Several hypotheses on the mechanism for $\Delta\bar{\mu}_{H^+}$-consuming ATP synthesis or $\Delta\bar{\mu}_{H^+}$-producing ATP hydrolysis catalyzed by $F_o \cdot F_1$-ATP synthase have been proposed and reviewed [8-13]. None of them is conclusively proved, and the molecular mechanism of coupling between proton flow through F_o and ATP synthesis on F_1 seems to be the most challenging problem in bioenergetics.

Three out of six nucleotides which can be cooperatively bound to one F_1 molecule are rather rapidly exchangeable with medium ATP or ADP [14, 15], thus suggesting that three sites on F_1 are potentially capable of participation in the catalytic steady-state turnovers during ATP hydrolysis or synthesis. The

Abbreviations: MF_1, CF_1, TF_1) solubilized F_1s from mitochondria, chloroplasts, and thermophylic bacteria, respectively; CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; AMP-PNP) 5′-adenylylimidodiphosphate; SMP) submitochondrial particles.

* To whom correspondence should be addressed.

0006-2979/99/6410-1128$22.00 ©1999 МАИК "Наука/Interperiodica"

presence of three exchangeable nucleotide-binding sites in addition to one or two P_i-binding site(s) [16] and several Me^{2+}-specific sites [17-21] per F_1 molecule gives rise to numerous possible enzyme–substrate (product) complexes which may exist during the steady-state catalysis in either direction. In contrast to several reports in the literature on the substrate–velocity curves for the complex [22-26], we found simple Michaelis–Menten dependence of the reaction rate on ATP concentration in the presence of Mg^{2+} [27] under the conditions where the true initial azide-insensitive steady-state rates of ATP hydrolysis were measured. Our results suggest that either a single nucleotide-binding site is involved in the catalysis or an extremely strong cooperativity such as a nucleotide binding exchange mechanism [28, 29] exists during the steady-state enzyme turnovers. It should be emphasized that the *true initial* rates of ATP hydrolysis (when no ADP is present in the assay system) are azide-insensitive, whereas the *apparent initial* rates (when the enzyme contains tightly bound ADP) are significantly decreased in the presence of azide [27].

The essential requirement of bivalent cations for ATP hydrolysis catalyzed by mitochondrial preparations of different degree of resolution was recognized many years ago [30-34]. However, an adequate answer to the simple question of the actual ATP- and Mg^{2+}-containing species participating as the substrate and activator during ATP hydrolysis has not been found. Numerous studies on this problem have appeared in the literature. It is generally believed that Mg·ATP is the substrate of F_1-type ATPase, whereas free ATP and free Mg^{2+} are inhibitors.

The standard kinetic analysis of enzymatic reactions involving metal–nucleotide complexes must be applied to F_1-type ATPase with great precautions for several reasons. When ATP hydrolysis is followed by continuous monitoring methods, the release of product is biphasic or triphasic depending on the "history" of the preparation. It shows a "burst" or "lag" on the minute time scale, and the time dependence for active $\rightarrow$ inactive or inactive $\rightarrow$ active transitions is a complex function of the nucleotides and Mg^{2+} concentrations [27, 35, 36]. Thus the possibility of reaching erroneous conclusions from measurements of apparent "initial" rates can hardly be overestimated. Another difficulty in the kinetic analysis of F_1-type ATPase is the possible presence of a small inhibitory peptide [37] which seems to be an intrinsic part of the mitochondrial $F_o \cdot F_1$-ATP synthase. The inhibitory effect of this protein is also time- and ATP-dependent in addition to the strong pH and ionic strength dependences [37, 38]. It appears that the presence of uncontrolled contaminations of the protein inhibitor might be a source of serious errors in the measurements of the actual rates of ATP hydrolysis, especially in early studies including our own [30-33]. The third difficulty is concerned with the strong requirement of Mg^{2+} for pyruvate kinase [39] which is routinely used for removal of inhibitory ADP in the coupled enzyme assay [40]. Thus variations of free Mg^{2+} in the continuous coupled assay mixtures are limited.

Our search of the literature reveals no report on the kinetic properties of $F_o \cdot F_1$ (which may or may not be identical to those of energetically uncoupled F_1) conclusively answering the question of whether ATP or Mg·ATP or Mg·ATP and Mg^{2+} serve as the substrate(s) of ATPase. In this paper we present initial rate analysis which shows that the Mg·ATP complex is the substrate for the $F_o \cdot F_1$-ATPase and the second Mg^{2+} bound in pH-dependent fashion is required for the catalysis. The data are suggestive of an ordered reaction mechanism where free Mg^{2+} participates in the reaction as an uncompetitive (with Mg·ATP complex) activator.

MATERIALS AND METHODS

Submitochondrial particles (SMP). SMP freed of the ATPase inhibitor were protein were prepared from bovine heart mitochondria as described [41] and stored as a suspension (small samples) in 0.25 M sucrose (30-40 mg/ml) in liquid nitrogen. Before the experiments a sample was thawed and diluted to a protein content of 5-10 mg/ml in a mixture containing (final concentrations) 0.25 M sucrose, 0.1 M KCl, 50 μM EDTA, 10 mM Hepes/KOH, and 5 mM potassium phosphate, pH 7.4. The suspension was incubated at 20°C for 1 h and further stored at room temperature (~20°C) during the experiments. The particles treated as described showed no lag in their initial azide-insensitive ATPase activity [42, 43] which was more than 95% inhibited by oligomycin (0.5 nmole/mg protein).

ATPase assay. ATPase activity was measured at 25°C in the standard reaction mixture containing (final concentrations) 0.25 M sucrose, 0.1 M KCl, 10 mM Hepes/KOH buffer, pH 7.4, 3 μM rotenone, 4 μM CCCP, and various concentrations of ATP and $MgCl_2$. The pH of the reaction mixture was adjusted after ATP and $MgCl_2$ were added. The pH was 8.0 when La·ATP was used as the substrate.

When the calculated free Mg^{2+} concentration was higher than 10^{-4} M, the activity was followed as a decrease in the absorption of NADH (160 μM) at 340 nm; 1.5 mM phosphoenolpyruvate (tricyclohexylammonium salt), pyruvate kinase (12 units/ml), and lactate dehydrogenase (10 units/ml) were added to the standard mixture, and the reaction was initiated by the addition of SMP (15-25 μg/ml).

When the free Mg^{2+} concentration was lower than 10^{-4} M, ATP hydrolysis was followed by "stoichiometric" H^+ release [44] as a decrease in the absorption of Phenol Red ($\Delta A_{557\text{-}620\ nm}$). Phenol Red (30 μM) was

added to the standard reaction mixture and the reaction was initiated by the addition of SMP (30-40 μg/ml). The stoichiometry of ADP formed/H^+ released which is dependent on pH and Mg^{2+} concentration [44] was determined as follows. After the reaction followed at 557-620 nm proceeded for 1-2 min, the ATP hydrolysis was stopped by addition of oligomycin (12 nmoles/ml) and the instrument sensitivity was calibrated by the additions of titrated HCl solution. The amount of ADP formed was then determined in the same sample by measuring of the absorption decrease at 340 nm after the addition of 160 μM NADH, 5 mM $MgCl_2$, 1.5 mM phosphoenolpyruvate, pyruvate kinase (12 units/ml), and lactate dehydrogenase (12 units/ml). It was shown in separate experiments that the pH indicator responded linearly to HCl concentration added and no absorption change of Phenol Red was observed at 340 nm when a pH shift of 0.05 unit was induced by HCl addition. All the activity measurements were performed using a Hitachi-557 spectrophotometer.

It should be noted that all the data and conclusions reported in this paper are valid only for the initial, constant (<30 sec) azide-insensitive rates [42].

Preparation of La·ATP. Equal volumes of 5 mM solutions of $LaCl_3$ and ATP (pH 3.0) were mixed, and the supernatant after centrifugation (to separate a precipitate formed) was used. The concentration of La·ATP was determined by the absorption at 259 nm. No turbidity was observed when the complex thus prepared was added to the assay mixture.

Estimation of ATP, Mg^{2+}, and Mg·ATP concentrations. The concentrations of the variable substrate (or activator or inhibitor) were calculated by solving for the positive roots of the polynomial equations derived from the multiple equilibria:

$$K_{Mg\cdot ATP} = \frac{[Mg^{2+}][ATP^{4-}]}{[Mg\cdot ATP^{2-}]};$$

$$K_{Mg\cdot ADP} = \frac{[Mg^{2+}][ADP^{3-}]}{[Mg\cdot ADP^{-}]};$$

$$K_{Ca\cdot ATP} = \frac{[Ca^{2+}][ATP^{4-}]}{[Ca\cdot ATP^{2-}]};$$

$$K_{Mg\cdot EDTA} = \frac{[Mg^{2+}][EDTA^{4-}]}{[Mg\cdot EDTA^{2-}]};$$

$$K_{Mg\cdot Cit} = \frac{[Mg^{2+}][Cit^{3-}]}{[Mg\cdot Cit^{1-}]},$$

where Cit stands for citrate, and the conservation equations for the total (analytical) concentrations of added Mg^{2+} ($[Mg]_t$), ATP^{4-} ($[ATP]_t$), $EDTA^{4-}$ ($[EDTA]_t$), ADP^{3-} ($[ADP]_t$), and citrate ($[Cit]_t$) were used. The dissociation constants $K_{Mg\cdot ATP}$ ($1\cdot10^{-4}$ M), $K_{Mg\cdot EDTA}$ ($1.6\cdot10^{-6}$ M), $K_{Mg\cdot ADP}$ ($1\cdot10^{-3}$ M), $K_{Mg\cdot Cit}$ ($2.5\cdot10^{-4}$ M), and $K_{Ca\cdot ATP}$ ($2.5\cdot10^{-4}$ M) corrected for pH 7.4 [45, 46] were used for the calculations. All calculations were performed using the GIM version 2 computer program (copyrighted by A. Drachev). It should be noted that slight variations in the absolute values for the dissociation constants found in the literature do not change our conclusions, although proportional variations in the values of $K_{m(i)}$ for substrate (or activator or inhibitor) are expected.

Reagents. ATP (sodium salt), lactate dehydrogenase, Hepes, EDTA, $NADP^+$, CCCP, oligomycin, and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (USA). Phosphoenolpyruvate (tricyclohexylammonium salt), pyruvate kinase, and NADH were from Reanal (Hungary). Hexokinase and rotenone were from Ferak (Germany), Chelex-100 was from Bio-Rad (USA). Other chemicals were of the purest grade commercially available.

The stock solutions of ATP and standard reaction mixture (when no EDTA or citrate were added) were treated with Chelex-100 ion-exchange resin (potassium form) to remove contaminating bivalent cations. The concentration of ATP in stock solutions was determined enzymatically ($NADP^+$, glucose, glucose-6-phosphate dehydrogenase). The concentration of Mg^{2+} in stock solutions was determined by titration with the standard EDTA in the presence of Eriochrome Black T in NH_4OH/NH_4Cl buffer (pH 10). The enzymes used for the analyses and assays were extensively dialyzed before use. The protein content was determined by the biuret method [47].

RESULTS

Mg·ATP as the substrate for $F_o\cdot F_1$-ATPase. No measurable ATP hydrolysis was observed in the absence of added Mg^{2+}. At fixed added ATP concentrations the ATPase activity (initial rates) increased as the concentration of added Mg^{2+} increased, and no inhibition of the reaction was seen up to the ratio $[Mg]_t/[ATP]_t$ of ~30 (Fig. 1a). The dependence of the initial rate on the added ATP concentration at constant $[Mg^{2+}]_t$ is shown in Fig. 1b. When the data were plotted as a function of calculated Mg·ATP complex, a single straight line was obtained (Fig. 1c), suggesting simple Michaelis–Menten kinetics of the reaction where Mg·ATP complex is the true substrate ($K_m^{Mg\cdot ATP}$ = 0.15 mM; V_{max} = 6.7 μmoles/min per mg protein). These results show that free ATP^{4-} (in the presence of Mg^{2+}) is neither a sub-

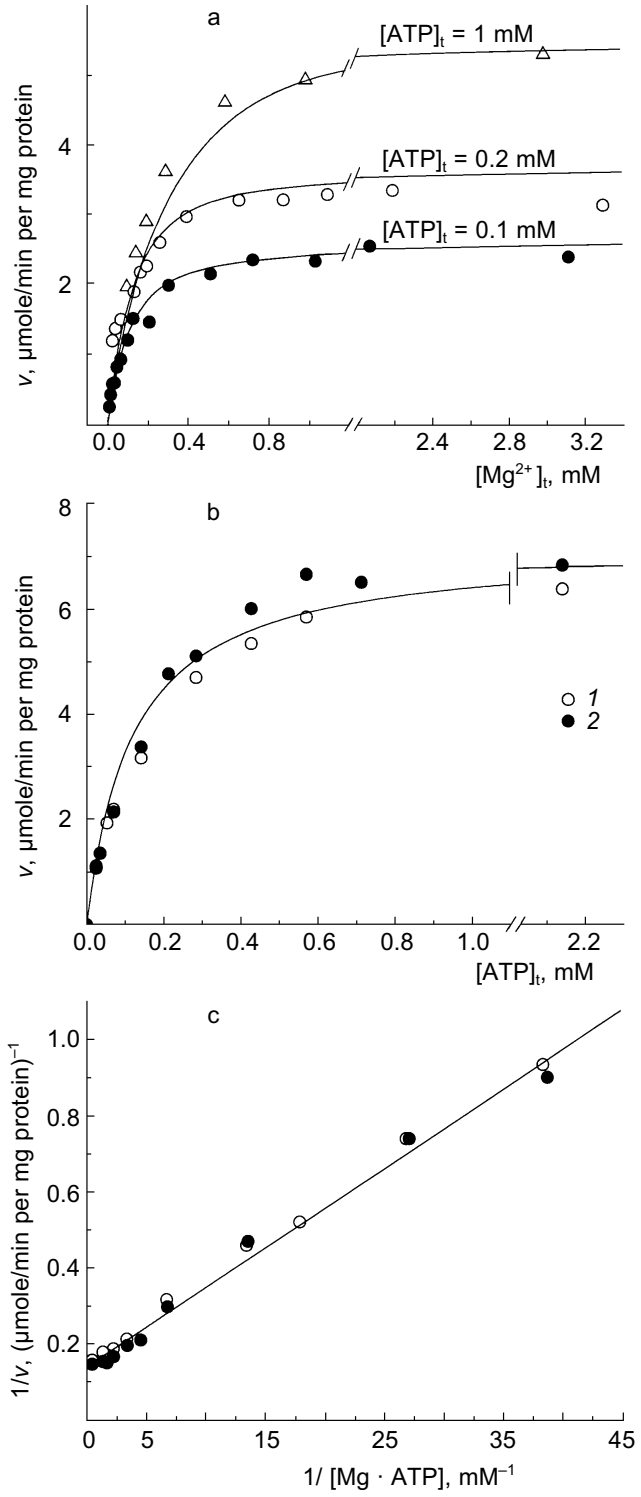

Fig. 1. Dependence of the initial rate of ATP hydrolysis on Mg^{2+} and ATP concentrations. a) Variations of $[Mg^{2+}]_t$ at constant concentrations of added ATP (indicated by the figures on the curves). The ATPase activity was assayed with ATP-regenerating system when $[Mg^{2+}]_t > [ATP]_t$ and by H^+ release when $[Mg^{2+}]_t < [ATP]_t$. b) Varying $[ATP]_t$ at 5 (*1*) and 10 mM $[Mg^{2+}]_t$ (*2*). c) The double reciprocal plot of the combined data presented in (a, △) and (b, ○ and ●) recalculated assuming that Mg·ATP is the substrate for hydrolysis.

strate or inhibitor of ATP hydrolysis. In agreement with our previous finding [27], we were unable to demonstrate any deviation from a simple hyperbolic dependence of the initial rate on the substrate concentration. No hydrolysis of ATP was observed in the presence of Ca^{2+}. At limited concentration of Mg·ATP ($\sim K_m^{Mg \cdot ATP}$), Ca^{2+} inhibited the reaction as it would be quantitatively expected if the inhibition were due to redistribution between catalytically inert Ca·ATP and active Mg·ATP complexes (table).

Binding of free Mg^{2+} is needed for hydrolysis of Mg·ATP. When added ATP was a variable parameter with a limited concentration of Mg^{2+}, the ATPase activity reached a maximum at the ratio $[ATP]_t/[Mg^{2+}]_t$ of approximately 1 and decreased upon further increase of free ATP (Fig. 2). The ascending parts of the curves are consistent with the results presented in the previous section suggesting that Mg·ATP is the substrate for the hydrolysis. At least two reasons for the descending parts of the curves may be considered. The possibility that free ATP^{4-} is an inhibitor of the reaction seemed to be unlikely in light of the results presented in Fig. 1c. An inhibition of the reaction at $[ATP]_t/[Mg^{2+}]_t >> 1$ would be also expected if the presence of free Mg^{2+} is needed for the hydrolysis of Mg·ATP. To test the latter explanation the buffer system containing Mg^{2+}, ATP, and other anions capable of Mg^{2+} binding was used to create a system in which concentration of the substrate (Mg·ATP) would be maintained at a constant level upon variation of free Mg^{2+}. For instance, if the reaction mixture contained 1.65 mM EDTA and 1 mM Mg^{2+} (total concentrations) the variation in $[ATP]_t$ from 0.2 to 1 mM results in

Apparent inhibition of $F_o \cdot F_1$-ATPase by Ca^{2+}

$[Ca^{2+}]$ added, mM	[Mg·ATP] present, mM	ATPase activity, %	
		expected	measured
0.00	0.18	100	100
0.58	0.14	90	91
1.15	0.12	83	85
2.30	0.09	71	72
4.60	0.06	55	58
11.50	0.03	34	39

* ATPase activity was measured as H^+-release (see "Materials and Methods" section). 100% correspond to the activity calculated using the parameters: V_{max} = 6.7 μmoles/min per mg protein and $K_m^{Mg \cdot ATP}$ = 0.15 mM. The concentrations of Mg·ATP were calculated by solving the cubic equation derived from the equilibria between ATP, Mg^{2+}, and Ca^{2+} at pH 7.4. The expected rates were calculated assuming a simple Michaelis–Menten dependence of the velocity on [Mg·ATP] and inability of Ca·ATP binding and hydrolysis at the catalytic site. $[ATP]_t$ and $[Mg^{2+}]_t$ were 0.2 and 1 mM, respectively.

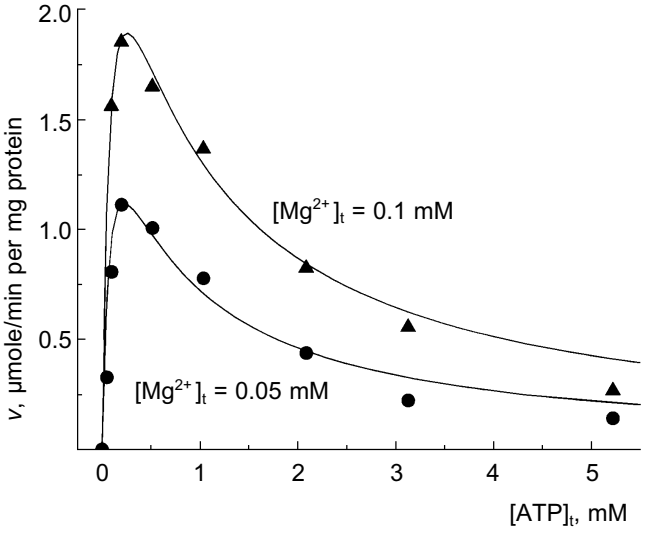

Fig. 2. Dependence of the initial rate of ATP hydrolysis on $[ATP]_t$ at fixed added Mg^{2+} concentrations. Concentrations of added Mg^{2+} are indicated on the curves. Symbols (●, ▲), the experimental points; continuous lines, the theoretical values calculated according Eq. (1) with the parameters: V_{max} = 6.1 μmoles/min per mg protein; $K_s^{Mg\cdot ATP}$ = 0.1 mM; and K_s^{Mg} = 0.025 mM.

variation of [Mg·ATP] from 4.7 to 23 μM, whereas the concentration of free Mg^{2+} remains almost constant (~2.4 μM). These complex buffer systems allow varying the substrate concentration at a particular constant free $[Mg^{2+}]$.

Figure 3 presents an example of the dependences of the reaction rate on the substrate concentrations ([Mg·ATP]) at different constant $[Mg^{2+}]$. When apparent maximal rates extrapolated to infinite [Mg·ATP] were plotted as a function of free Mg^{2+} concentrations, a simple hyperbolae was obtained with the parameters: K_m^{Mg} = 0.02 mM and V_{max} = 6.3 μmoles/min per mg protein (Fig. 3a). It appears that a single activating Mg^{2+}-specific site is operating during the steady-state catalysis. The kinetic behavior of the reaction (Fig. 3b) suggests the formal ping-pong mechanism, where the substrate (Mg·ATP) transformation at the enzyme active site (hydrolysis of Mg·ATP and/or release of one product (ADP or P_i)) precedes the involvement of free Mg^{2+}, which is needed to complete the catalytic cycle. The kinetic equation for such a mechanism (see scheme shown in Fig. 8 in the "Discussion" section) appears as:

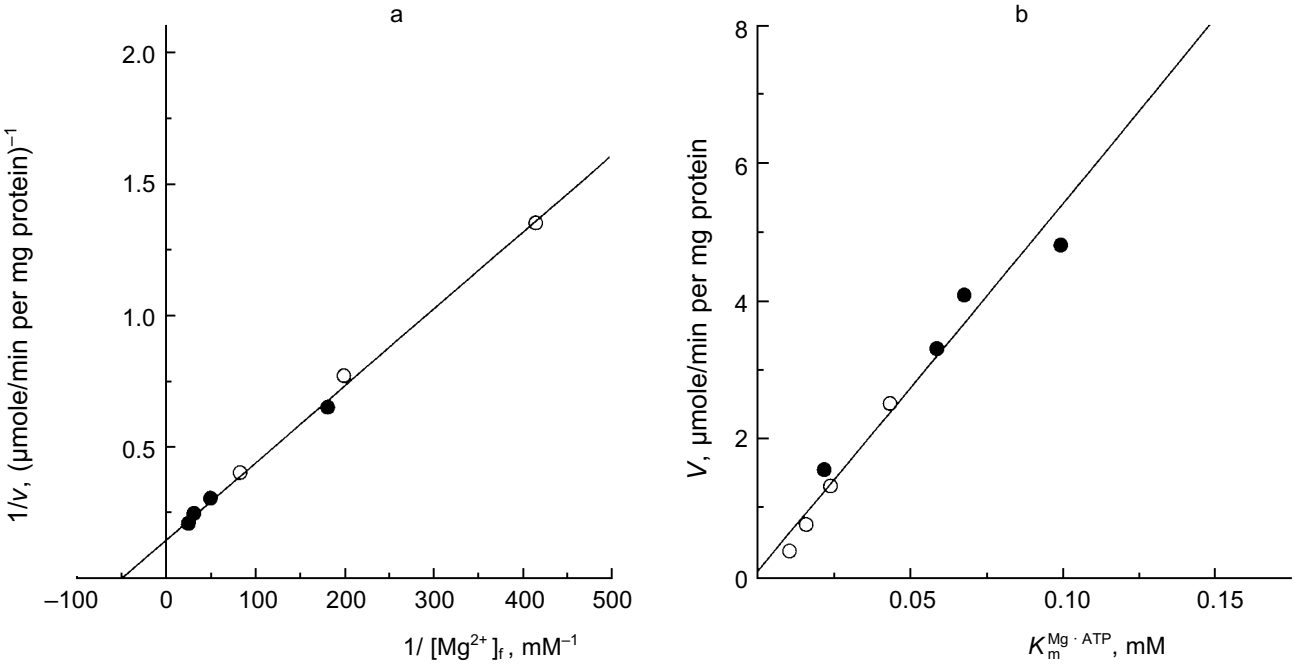

Fig. 3. Kinetic parameters of Mg·ATP hydrolysis at different Mg^{2+} concentrations. a) The reaction rates (v) extrapolated to infinite Mg·ATP were measured as a function of free Mg^{2+} (double reciprocal plot). Concentrations of constant free Mg^{2+} were maintained utilizing Mg^{2+}/citrate/ATP buffer (○) or Mg^{2+}/EDTA/ATP buffer (●) (see "Materials and Methods" section). b) Secondary plot (V_{max} versus $K_m^{Mg\cdot ATP}$) of data obtained as in (a).

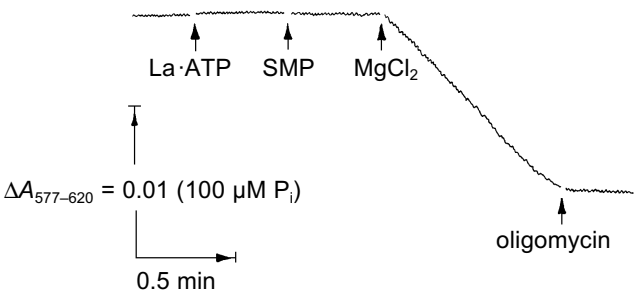

Fig. 4. Hydrolysis of La·ATP by $F_o \cdot F_1$-ATPase. The reaction was followed as H^+ release measured with Phenol Red (see "Materials and Methods" section). La·ATP (0.2 mM), $MgCl_2$ (0.2 mM), SMP (0.4 mg/ml), and oligomycin (12 nmoles/ml) were added where indicated by the arrows. The rate of La·ATP hydrolysis initiated by the addition of Mg^{2+} was 0.7 μmole/min per mg protein.

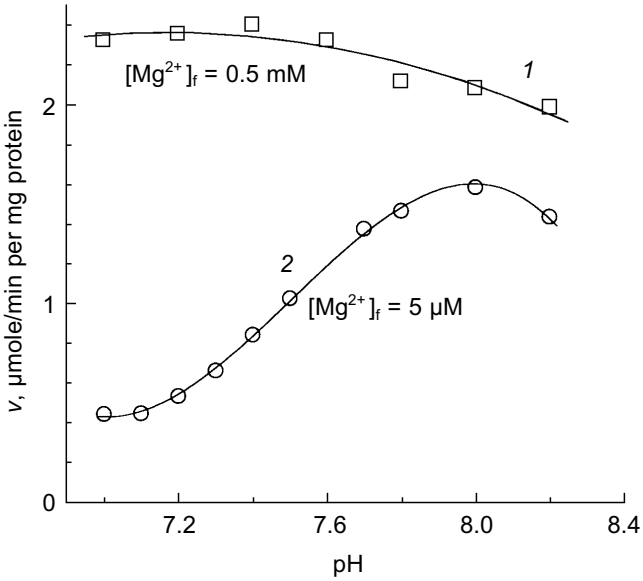

Fig. 5. pH dependence of ATPase activity. *1*) Mg^{2+} concentration was 0.5 mM; ATP-regenerating system was used for ATPase assay; *2*) Mg^{2+} concentration was 5 μM; ATPase activity was assayed as H^+ release. H^+ released/ADP formed ratio was varied from 0.68 at pH 7.0 to 0.96 at pH 8.2 and the rates were corrected as described in "Materials and Methods" section.

$$v = (k_{cat} \cdot [E_t] \cdot [Mg{\cdot}ATP] \cdot [Mg^{2+}]) /$$

$$/ ((1 + k_{cat}/k_p) \cdot [Mg{\cdot}ATP] \cdot [Mg^{2+}] +$$

$$+ K_s^{Mg} \cdot [Mg{\cdot}ATP] + K_s^{Mg{\cdot}ATP} \cdot [Mg^{2+}] \cdot k_{cat}/k_p); \quad (1)$$

and it is expected that an inhibition of the reaction would occur at high concentrations of added ATP at any given Mg^{2+} concentration. Figure 2 shows that the experimentally determined initial rates fit the theoretical prediction when the approximated values of $K_s^{Mg{\cdot}ATP}$ and K_s^{Mg} were used. Taken together, the results presented in this and previous section strongly suggest that Mg·ATP is a true substrate for $F_o \cdot F_1$-ATPase and free Mg^{2+} is needed as an obligatory activator for the steady-state ATP hydrolysis.

It should be noted that an alternative interpretation of the data shown in Fig. 2 is possible assuming that Mg·ATP is the substrate and free ATP is an inhibitor. An ambiguity in interpretation is due to the fact that the concentrations of ATP and Mg^{2+} at equilibrium are certain to obey the mass action low. It was desirable to find a more direct experimental approach to show the requirement of free Mg^{2+} for the ATPase reaction. The kinetically inert complexes of metals with ATP [48, 49] seemed to be a useful tool to discriminate between the two possible interpretations. This approach was used previously by Pedersen's group for the soluble rat liver F_1 [48]. As depicted in Fig. 4, the La·ATP complex is not hydrolyzed unless Mg^{2+} is added. In the presence of free Mg^{2+} the rate of ATP hydrolysis is about 15% of that observed for the Mg·ATP maximal rate.

Since the affinity of the binding site for a cation is expected to be pH dependent, it was of interest to determine how the pH dependence of the enzyme activity (if it exists) is related to the Mg^{2+} requirement. Figure 5 demonstrates that the initial rate of ATP hydrolysis was only slightly changed within the pH interval from 6.8 to 8.0 when free Mg^{2+} was in excess (~10 K_m^{Mg}) and becomes strongly pH dependent when free Mg^{2+} was limiting. An apparent pK_a of 7.4 for a group or groups involved in Mg^{2+} binding was esti-

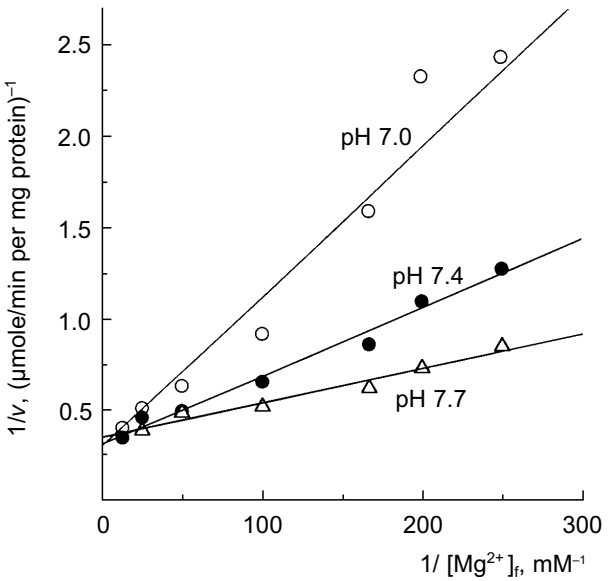

Fig. 6. Competitive inhibition of ATPase by H^+. [Mg·ATP] was 0.1 mM; calculated reciprocal free concentrations of Mg^{2+} are plotted on the abscissa.

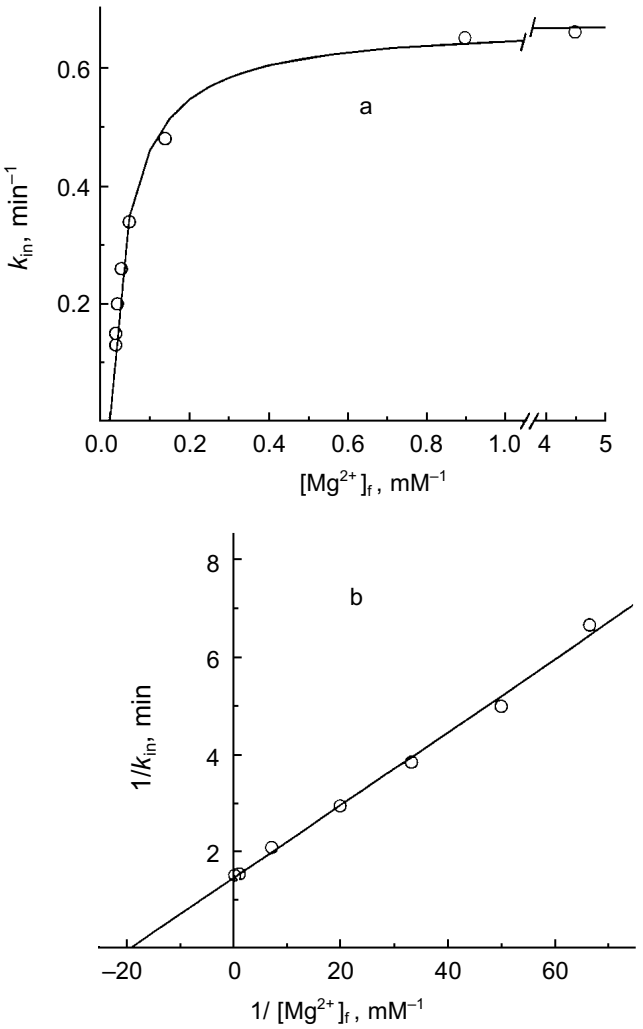

Fig. 7. Mg^{2+} dependence of inhibition of ATPase by azide. a) The standard reaction mixture contained 200 μM sodium azide and increased amount of pyruvate kinase (300 units/ml) to avoid rate limitation at low free [Mg^{2+}]. The concentration of Mg·ATP was 1 mM. The reaction was initiated by the addition of SMP and followed continuously. The first-order rate constant of azide-induced inhibition was calculated [42] and plotted as a function of free [Mg^{2+}]. Concentrations of free Mg^{2+} were varied by the excess of ATP added. b) Double reciprocal plot of the data shown in (a).

mated from the titration curve, and simple competition was observed between activating free Mg^{2+} and H^+ (Fig. 6).

Mg^{2+}(ADP)-specific time-dependent inhibition and requirement of free Mg^{2+} for ATP hydrolysis. It has been shown that ADP-loaded $F_o{\cdot}F_1$-ATPase is sensitive to Mg^{2+}-induced slow isomerization of the ADP–enzyme complex, producing inactive ATPase which is stabilized by azide [42, 50]. Since the binding of free Mg^{2+} at the enzyme specific site was shown to be required for the steady-state ATP hydrolysis (see previous section), it was of interest to get deeper insight into the relations between the role of Mg^{2+} in the catalysis and in the deactivation process. The parameters of ATP hydrolysis activation by Mg^{2+} (Fig. 3a) and those determined as the first-order rate constant for the azide-induced deactivation [42] (Fig. 7) were quantitatively estimated. Both dependences were found to fit a simple hyperbolic function with the dissociation constant for Mg^{2+} of about $4{\cdot}10^{-5}$ M. These results are in accord with the proposal on a single free Mg^{2+} binding site.

DISCUSSION

Many years ago we reported that depending on the "history" of the preparation the mitochondrial F_1- or $F_o{\cdot}F_1$-ATPase shows either lag-phase or burst in the activity assayed in the presence of an ATP-regenerating system [27]. The interplay of Mg^{2+}-dependent deactivation of the enzyme–ADP intermediate [27, 50] and ATP-dependent reactivation of the inhibited enzyme [27] (both reactions are slow compared with the enzyme turnover) could lead to erroneous interpretation of the velocity versus substrate curves. Moreover, the meaning of the "initial rate", which is a standard parameter used for steady-state kinetic analysis, becomes ambiguous when applied to a system where the amount of catalytically competent enzyme is changed within the time scale comparable with the resolution limit of the assay. These considerations prompted us to reinvestigate a long standing problem: what are the true species which interact with $F_o{\cdot}F_1$-ATPase during steady-state ATP hydrolysis? Our data confirm and reinforce the generally accepted belief (for exceptions, see [51, 52]) that Mg·ATP complex is the specie taken up from solution as the substrate for the reaction. However, our results reject another generally accepted view, that free Mg^{2+} is an inhibitor of the reaction [34]. The most likely reason for the latter is that no precautions for complete activation of ATPase (removal of tightly bound ADP [27]) were taken in the previously published works. When the concentration of free Mg^{2+} in the assay system is high, a fraction of the enzyme preparation becomes rapidly deactivated, and the apparent "initial rate" is underestimated.

Our data show that free Mg^{2+} (in addition to Mg·ATP) is needed for the catalysis. This is in accord with the conclusion reached by other authors for F_1 from rat liver [48] and from *Halobacterium saccharovorum* [53]. In addition to providing new information about the participation of free Mg^{2+} in Mg·ATP hydrolysis by $F_o{\cdot}F_1$-ATPase, the results presented here are relevant to the sequence of the catalytic steps. The extended kinetic scheme of the reaction is shown in Fig. 8. As shown in this study, the activating effect of free Mg^{2+} is described by the formal ping-pong mechanism.

Mechanistically this would mean that activating Mg^{2+} participates in the overall reaction after one of the products (ADP or P_i) is released. Several findings suggest that P_i leaves the ATPase active site before ADP. First, it is well documented that P_i is not required for ADP binding. Moreover, P_i reverses ADP-induced (in the presence of Mg^{2+}) deactivation of the ATPase [43] or Mg^{2+}-induced deactivation of the ADP-preloaded ATPase [50]. Second, both the steady-state rate of ATP hydrolysis (routes (1)-(5)) and the deactivation rate (routes (1)-(6)) are changed proportionally when either Mg·ATP [27] or free Mg^{2+} (this paper) are variable parameters, independently of the presence of P_i. The absence of any inhibitory effect of P_i on $F_o \cdot F_1$-ATPase [43] suggests that P_i leaves the enzyme during uncoupled ATP hydrolysis from a very low-affinity site. The low affinity for leaving P_i compared to the high affinity for ADP [35] can provide a thermodynamic basis for the kinetic sequence of the release of the products during ATP hydrolysis. It worth noting that the apparent K_m value for P_i during oxidative phosphorylation under conditions similar to those used in this work for ATPase assay is about 1 mM (our unpublished observation). The absence of the inhibitory effect of P_i on the ATPase is hard to reconcile with simple reversibility of the reaction steps during hydrolysis and synthesis of ATP.

Strong data for the presence of a Mg^{2+}-specific site on mitochondrial $F_o \cdot F_1$-ATPase [50] and CF_1 [55] which is responsible for Mg^{2+}-induced deactivation of ATPase have been reported. A two-step mechanism for Mg^{2+}-induced deactivation of ADP-loaded enzyme was proposed, which includes rapid, pH and nucleotide-dependent binding of Mg^{2+} (K_s value of $1 \cdot 10^{-4}$ M at pH 7.4) and subsequent isomerization of the complex resulting in the inactive enzyme [50]. The apparent affinities for free Mg^{2+} determined from quantitation of: (1) Mg^{2+}-induced deactivation of ADP-preloaded $F_o \cdot F_1$ [50], (2) Mg^{2+} requirement of Mg·ATP hydrolysis (this paper), and (3) Mg^{2+} dependence of azide-induced inhibition (this paper) at pH 7.4 are $1 \cdot 10^{-4}$, $2 \cdot 10^{-5}$, and $4 \cdot 10^{-5}$ M, respectively. The latter two values are similar, and it is not unexpected that the value determined for (1) is considerably higher: a strong dependence of the apparent affinity for deactivating Mg^{2+} on occupation of nucleotide-binding sites has been documented [21]. The actual occupancy of three rapidly exchangeable nucleotide-binding sites during steady-state ATP hydrolysis is not known. Thus, we believe that the same Mg^{2+}-specific site is responsible for all three processes, and the reaction sequences (3)-(4)-(5) and (3)-(4)-(6) (Fig. 8) describe the final steps of ATP hydrolysis and deactivation, respectively. The simple competitive inhibition of Mg·ATP hydrolysis by free ADP (our results which are not included in this paper) is expected if the reaction sequence proceeds as depicted in the reaction scheme whereby free ADP leaves the ATPase catalytic site via a Mg^{2+}-dependent mechanism. The tight binding of ADP and some nucleotide derivatives to TF_1 and MF_1 in the absence of Mg^{2+} and major

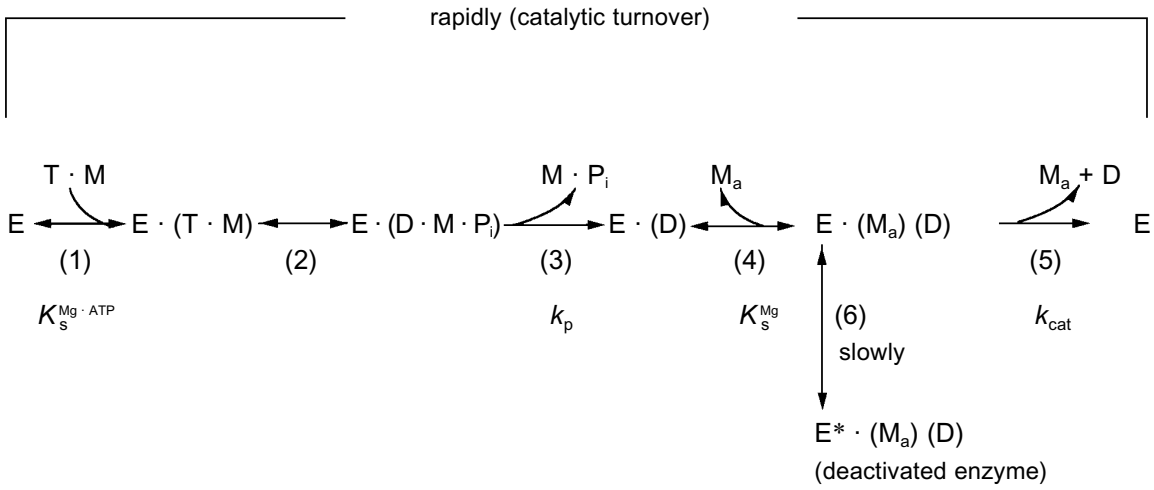

Fig. 8. Reaction scheme for hydrolysis of ATP catalyzed by the mitochondrial $F_o \cdot F_1$-ATPase. E, T, D, P_i, M, and M_a stand for the enzyme, ATP, ADP, inorganic phosphate, Mg^{2+} originated from Mg·ATP, and free activating Mg^{2+}, respectively. The scheme does not attempt to portray the interactions between the enzyme subunits during the reaction. The hydrolytic steps (2)-(5) may certainly depend on the cooperative interactions within multisubunit enzyme (binding change mechanism [12]) possibly including rotation of the γ-subunit [54]. The scheme is rather intended to describe the fate of the substrate during catalytic turnover. It should be noted that steps (4) and (5) are shown as binding and release of Mg^{2+} for the sake of clarity; no difference in the kinetic behavior of the system (Eq. (1)) would be seen if the activating Mg^{2+} (M_a) remains permanently bound to the enzyme during the catalysis, participating in the reaction cycle after P_i release. For simplicity, a combination of equilibrium (steps (1)-(2) and (4)) and steady-state (steps (3) and (5)) approximations were applied to derive Eq. (1) describing the reaction scheme.

rearrangements of the nucleotide-binding sites after subsequent addition of Mg^{2+} have been documented [56, 57]. On the other hand, Mg·ADP serves as the substrate for photo- [58] or oxidative phosphorylation (our unpublished results). The difference in the reactive species leaving the catalytic site during steady-state ATP hydrolysis and entering the catalytic site during steady-state ATP synthesis is in accord with our hypothesis of different catalytic mechanisms for operation of $F_o{\cdot}F_1$ in the hydrolytic and synthetic directions [59, 60].

Since the structure at 2.8 Å resolution of bovine heart mitochondrial F_1-ATPase became available [5], an obvious question arises: where is the single Mg^{2+}-specific site located? Structures of the nucleotide-binding sites of asymmetric F_1 containing Mg^{2+}–nucleotide complexes with a slight difference in the ligands to Mg^{2+} in the α- and β-subunits were identified [5]. Surprisingly, no Mg^{2+} other than that originating from the Mg^{2+}–nucleotide complexes was visualized despite the presence of 250 μM AMP-PNP, 5 μM ADP, 6 mM Mg^{2+}, and 3 mM sodium azide and the absence of P_i in the crystallization medium [61], i.e., conditions which must produce the deactivated Mg^{2+}-containing enzyme. Several possibilities for an explanation of the absence of visualized Mg^{2+} can be offered. Major rearrangement of F_1 subunits and the substrate/activator binding sites induced by interaction of F_1 and F_o is one possible reason [62]. Another possibility is that the Mg^{2+}-specific site is located on a disordered "invisible" part of the γ-subunit. Such speculation may be supported by the findings that ATP hydrolysis catalyzed by the reconstituted $3\alpha{\cdot}3\beta$ TF_1 is not sensitive to azide [63, 64], which is known to inhibit ATPase by a Mg^{2+}-dependent mechanism ([21, 42, 50] and this paper). If this hypothesis is correct, the structural arrangements of the events shown in the reaction scheme (Fig. 8) are that free ADP formed at the catalytic site after ($P_i{\cdot}Mg$) release is removed to the solution via the interaction between the Mg^{2+}-liganded arm of the γ-subunit and the β-subunit. A key role of Mg^{2+} in the energy transducing enzymes has long been suggested [65, 66]. The large pH dependence of the Mg^{2+}-specific site evidenced from the Mg^{2+}-dependent steady-state hydrolysis of Mg·ATP (Figs. 5 and 6) is in line with this proposal. The prediction of the hypothesis on the key role of Mg^{2+} in coupling between transmembrane proton or sodium [67] flows and binding/release of nucleotides at the catalytic site(s) is that the binding of Mg^{2+} to the F_1 part of $F_o{\cdot}F_1$-ATPase/synthase must be $\Delta\bar{\mu}_{H^+}$-dependent. Experiments aimed to clarify this possibility are currently underway in our laboratory.

We thank Drs. E. Maklashina, V. Grivennikova, and E. Gavrikova for their help in the preparation of SMP.

This work was supported by the Russian Foundation for Basic Research (grants 96-04-48185 and 99-04-48082) and by the Program for Advanced Schools in Science (grant 96-15-97822).

REFERENCES

1. Catteral, W. A., and Pedersen, P. L. (1971) *J. Biol. Chem.*, **246**, 4987-4994.
2. Kagawa, Y., Sone, N., Yoshida, M., Hirata, H., and Okamoto, H. (1976) *J. Biochem.* (Tokyo), **80**, 141-151.
3. Foster, D. L., and Fillingame, R. H. (1979) *J. Biol. Chem.*, **254**, 8230-8236.
4. Penefsky, H. S., Pullman, M. E., Datta, A., and Racker, E. (1960) *J. Biol. Chem.*, **235**, 3330-3336.
5. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature* (London), **370**, 621-628.
6. Foster, D. L., and Fillingame, R. H. (1982) *J. Biol. Chem.*, **257**, 2009-2015.
7. Collinson, I. R., Runswick, M. J., Buchanan, S. K., Fearnley, I. M., Skehel, J. M., van Raaij, M. J., Griffiths, D. E., and Walker, J. E. (1994) *Biochemistry*, **33**, 7971-7978.
8. Mitchell, P. (1961) *Nature* (London), **191**, 144-148.
9. Mitchell, P. (1974) *FEBS Lett.*, **43**, 189-194.
10. Senior, A. E. (1988) *Physiol. Rev.*, **68**, 177-231.
11. Penefsky, H. S., and Cross, R. L. (1991) *Adv. Enzymol.*, **64**, 173-214.
12. Boyer, P. D. (1993) *Biochim. Biophys. Acta*, **1140**, 215-250.
13. Pedersen, P. L., and Amzel, L. M. (1993) *J. Biol. Chem.*, **268**, 9937-9940.
14. Cross, R. L., and Nalin, G. M. (1982) *J. Biol. Chem.*, **257**, 2874-2881.
15. Kironde, F. A. S., and Cross, R. L. (1986) *J. Biol. Chem.*, **261**, 12544-12549.
16. Kasahara, M., and Penefsky, H. S. (1978) *J. Biol. Chem.*, **253**, 4180-4187.
17. Senior, A. E. (1979) *J. Biol. Chem.*, **254**, 11319-11322.
18. Williams, N., Hullihen, J., and Pedersen, P. L. (1987) *Biochemistry*, **26**, 162-169.
19. Haddy, A. E., Frasch, W. D., and Sharp, R. R. (1989) *Biochemistry*, **28**, 3664-3669.
20. Pedersen, P. L., Williams, N., and Hullihen, J. (1987) *Biochemistry*, **26**, 8631-8637.
21. Bulygin, V. V., Syroeshkin, A. V., and Vinogradov, A. D. (1993) *FEBS Lett.*, **328**, 193-196.
22. Ebel, R. E., and Lardy, H. A. (1975) *J. Biol. Chem.*, **250**, 190-196.
23. Schuster, S. M., Ebel, R. F., and Lardy, H. A. (1975) *J. Biol. Chem.*, **250**, 7848-7853.
24. Roveri, O. A., and Calcaterra, N. B. (1985) *FEBS Lett.*, **192**, 123-127.
25. Wong, S. Y., Matsuno-Yagi, A., and Hatefi, Y. (1984) *Biochemistry*, **23**, 5004-5010.
26. Harris, D. A. (1989) *Biochim. Biophys. Acta*, **974**, 156-162.
27. Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., and Vinogradov, A. D. (1982) *Biochem. J.*, **202**, 9-14.
28. Repke, K. R. H., and Schön, R. (1973) *Acta Biol. Med. Germ.*, **31**, K-19-K-30.
29. Rosing, J., Kayalar, C., and Boyer, P. D. (1977) *J. Biol. Chem.*, **252**, 2478-2485.
30. Cooper, C., and Lehninger, A. L. (1957) *J. Biol. Chem.*, **224**, 547-560.
31. Selwyn, M. J. (1968) *Nature* (London), **219**, 490-493.
32. Akimenko, V. K., Minkov, I. B., Bakeeva, L. E., and Vinogradov, A. D. (1972) *Biokhimiya*, **37**, 285-293.
33. Adolfsen, R., and Moudrianakis, E. (1973) *Biochemistry*, **12**, 2926-2933.

34. Fleury, B., Di Pietro, A., Godinot, C., and Gautheron, D. (1980) *Biochimie*, **62**, 733-737.
35. Vasilyeva, E. A., Fitin, A. F., Minkov, I. B., and Vinogradov, A. D. (1980) *Biochem. J.*, **188**, 807-815.
36. Vinogradov, A. D. (1972) *Biokhimiya*, **49**, 1220-1238.
37. Pullman, E. M., and Monroy, G. G. (1963) *J. Biol. Chem.*, **238**, 3762-3769.
38. Panchenko, M. V., and Vinogradov, A. D. (1985) *FEBS Lett.*, **184**, 226-230.
39. Gupta, R. K., Oesterling, R. M., and Mildvan, A. S. (1976) *Biochemistry*, **15**, 2881-2887.
40. Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960) *J. Biol. Chem.*, **235**, 3322-3329.
41. Kotlyar, A. B., and Vinogradov, A. D. (1990) *Biochim. Biophys. Acta*, **1019**, 151-158.
42. Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., and Vinogradov, A. D. (1982) *Biochem. J.*, **202**, 15-23.
43. Yalamova, M. V., Vasilyeva, E. A., and Vinogradov, A. D. (1982) *Biochem. Int.*, **4**, 337-344.
44. Chance, B., and Nishimura, M. (1967) *Meth. Enzymol.*, **10**, 641-650.
45. Shikama, K. (1971) *Arch. Biochem. Biophys.*, **147**, 311-317.
46. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1986) *Data for Biochemical Research*, Clarendon Press, Oxford.
47. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.*, **177**, 751-766.
48. Hanley-Trawick, S., Carpen, M. E., Dunaway-Mariano, D., Pedersen, P. L., and Hullihen, J. (1989) *Arch. Biochem. Biophys.*, **268**, 116-123.
49. Bossard, M. J., Vik, T. A., and Schuster, S. M. (1980) *J. Biol. Chem.*, **255**, 5342-5346.
50. Bulygin, V. V., and Vinogradov, A. D. (1991) *Biochem. J.*, **276**, 149-156.
51. Berger, G., Girault, G., Galmiche, J.-M., and Pezennec, S. (1994) *J. Bioenerg. Biomembr.*, **26**, 335-346.
52. Pezennec, S., Berger, G., Andrianambinintsoa, S., Radziszewski, N., Girault, G., Galmiche, J.-M., and Bäuerlein, E. (1995) *Biochim. Biophys. Acta*, **1231**, 98-110.
53. Schobert, B. (1992) *J. Biol. Chem.*, **267**, 10252-10257.
54. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. (1997) *Nature*, **386**, 299-302.
55. Guerrero, K. J., Xue, Z., and Boyer, P. D. (1990) *J. Biol. Chem.*, **265**, 16280-16287.
56. Yoshida, M., and Allison, W. S. (1983) *J. Biol. Chem.*, **258**, 14407-14412.
57. Burgard, S., Nett, J. H., Sauer, H. S., Kagawa, Y., Schäfer, H.-J., Wise, J. G., Vogel, P. D., and Tommer, W. E. (1994) *J. Biol. Chem.*, **269**, 17815-17819.
58. Zhou, J.-M., and Boyer, P. D. (1992) *Biochemistry*, **31**, 3166-3171.
59. Minkov, I. B., Vasilyeva, E. A., Fitin, A. F., and Vinogradov, A. D. (1980) *Biochem. Int.*, **1**, 478-485.
60. Syroeshkin, A. V., Vasilyeva, E. A., and Vinogradov, A. D. (1995) *FEBS Lett.*, **366**, 29-32.
61. Lutter, R., Abrahams, J. P., van Raaij, M. J., Todd, R. J., Lundqvist, T., Buchanan, S. K., Leslie, A. G. W., and Walker, J. E. (1993) *J. Mol. Biol.*, **229**, 787-790.
62. Böttcher, B., Gräber, P., Boekema, E. J., and Lücken, U. (1995) *FEBS Lett.*, **373**, 262-264.
63. Kagawa, Y., Ohta, S., and Otawara-Hamamoto, Y. (1989) *FEBS Lett.*, **249**, 67-69.
64. Miwa, K., and Yoshida, M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6484-6487.
65. Racker, E. (1977) *Annu. Rev. Biochem.*, **46**, 1006-1014.
66. Jenkins, W. T., Marshall, M. M., and Lewin, A. S. (1984) *Arch. Biochem. Biophys.*, **232**, 496-504.
67. Laubinger, W., and Dimroth, P. (1988) *Biochemistry*, **27**, 7531-7537.