ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 3, pp. 327-335. © Pleiades Publishing, Ltd., 2010.
Published in Russian in Biokhimiya, 2010, Vol. 75, No. 3, pp. 400-410.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM09-235, January 17, 2010.

Probes of Inhibition of *Escherichia coli* F_1-ATPase by 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole in the Presence of MgADP and MgATP Support a Bi-Site Mechanism of ATP Hydrolysis by the Enzyme

V. V. Bulygin[#] and Y. M. Milgrom*

Department of Biochemistry and Molecular Biology, State University of New York, Upstate Medical University, Syracuse, New York 13210, USA; fax: +1-315-464-8750; E-mail: milgromy@upstate.edu

Received July 30, 2009
Revision received September 18, 2009

Abstract—Binding of MgADP and MgATP to *Escherichia coli* F_1-ATPase (EcF_1) has been assessed by their effects on extent of the enzyme inhibition by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl). MgADP at low concentrations (K_d 1.3 μM) promotes the inhibition, whereas at higher concentrations (K_d 0.7 mM) EcF_1 is protected from inhibition. The mutant βY331W-EcF_1 requires much higher MgADP, K_d of about 10 mM, for protection. Such MgADP binding was not revealed by fluorescence quenching measurements. MgATP partially protects EcF_1 from inactivation by NBD-Cl, but the enzyme remains sensitive to NBD-Cl in the presence of MgATP at concentrations as high as 10 mM. The activating anion selenite in the absence of MgATP partially protects EcF_1 from inhibition by NBD-Cl. A complete protection of EcF_1 from inhibition by NBD-Cl has been observed in the presence of both MgATP and selenite. The results support a bi-site catalytic mechanism for MgATP hydrolysis by F_1-ATPases and suggest that stimulation of the enzyme activity by activating anions is due to the anion binding to a catalytic site that remains unoccupied at saturating substrate concentration.

DOI: 10.1134/S0006297910030090

Key words: anion activation, ATP synthase, catalytic cooperativity, bi-site catalysis, nucleotide binding, tri-site catalysis

F_1-ATPase is the catalytic component of F_oF_1-ATP synthase responsible for ATP synthesis during oxidative and photophosphorylation in eubacteria, mitochondria, and chloroplasts. F_1 consists of five types of subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ and is capable only of net ATP hydrolysis when separated from the membrane-bound factor F_o that is responsible for trans-membrane transport of H^+ (and, in some bacteria, Na^+) and in *Escherichia coli* is formed by *a* and *b* subunits and a ring of *c* subunits in stoichiometry ab_2c_{10}. In the high-resolution crystal structure of beef heart MF_1, α and β subunits are arranged alternatively in a hexamer around the γ subunit [1]. Six nucleotide-binding sites of F_1 [2-4] are differentiated functionally as three catalytic and three noncatalytic [2]. Catalytic and noncatalytic sites are located at alternating α/β interfaces and are formed primarily by the amino acid residues of β and α subunits, respectively [1]. Synthesis and hydrolysis of ATP coupled to transmembrane H^+-transport by F_oF_1 is best described by the rotary binding change mechanism proposed by Boyer and colleagues [5-7] and involves a rotation of a complex of subunits (rotor, $\gamma\varepsilon c_{10}$ in *E. coli*) relative to a stator composed of $\alpha_3\beta_3\delta ab_2$ [8, 9]. During a catalytic cycle, each of the three catalytic sites of F_1 sequentially proceeds through an identical set of conformational states in a series of tightly coordinated conformational transitions [10, 11].

As predicted by the binding change mechanism and first shown with MF_1, the enzyme turnover is slow and limited by product dissociation when only one catalytic site is occupied [12]. Such mode of enzyme turnover is referred to as uni-site catalysis [12]. MgATP binding at additional catalytic site(s) of MF_1 induces acceleration of product release resulting in positive cooperativity in

Abbreviations: F_1, solubilized portion of F_oF_1-ATP synthase; MF_1, EcF_1, and TF_1, F_1-ATPases from beef-heart mitochondria, *Escherichia coli*, and thermophilic *Bacillus* PS3, respectively; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole.

[#] Deceased.

* To whom correspondence should be addressed.

catalysis [12, 13]. Similar results have been subsequently obtained with EcF_1 [14]. Cooperative mode of F_1 turnover (multi-site catalysis) has been referred to as bi-site or tri-site catalysis depending on the presumed catalytic site occupancy [13]. Under conditions when formation of the inactive enzyme complex with MgADP bound at one of the catalytic sites [15] is avoided, MgATP concentration dependence of F_1 activity does not deviate from Michaelis–Menten behavior [16-22] even at submicromolar MgATP concentrations [21, 23, 24]. Similarly, MgATP-concentration dependence of the rate of γ-subunit rotation is hyperbolic over a very wide range of MgATP concentrations [21, 22].

These results strongly indicate that a single catalytic mechanism, either bi-site or tri-site catalysis, is responsible for multi-site ATP hydrolysis and ATP-induced γ-subunit rotation. Studies of the transition to multi-site catalysis during ATP synthesis by chloroplasts [25] and ATP hydrolysis by MF_1 [23], as well as studies on competition between TNP-ATP and ATP for binding to MF_1 [26] strongly support the bi-site model of multi-site catalysis. Additional evidence supporting the bi-site model has been recently provided by the results obtained when the catalytic site occupancy during multi-site ATP hydrolysis by MF_1 [27] and EcF_1 [24] has been measured using a centrifugal filtration method. A major part of experimental support for the tri-site model comes from the fluorescence studies first performed with βY331W-mutant EcF_1 [28] and subsequently with the homologous βY341W-mutant $\alpha_3\beta_3\gamma$-subcomplex of TF_1 [29, 30] and βY345W-mutant MF_1 from the yeast *Saccharomyces cerevisiae* [31]. In these studies, catalytic-site occupancy during ATP hydrolysis was estimated using nucleotide-induced quenching of fluorescence of the engineered tryptophan residues assuming the existence of proportionality between the extent of occupancy of all three catalytic sites and the extent of fluorescence quenching. The results were interpreted as supporting a tri-site model with the concentration of MgATP required for half-maximal occupancy of the third catalytic site and K_d values for MgADP binding at the third catalytic site to be less than 0.1 mM.

We have recently presented data showing that when βY331W-EcF_1 is titrated with ADP, ATP, or MgADP, the nucleotide concentration dependence of fluorescence quenching is biphasic with each phase contributing about equally to the overall nucleotide-induced quenching [32]. Based on these results, as well as on similar titration curves obtained by others when fluorescence of βY331W-EcF_1 was quenched by MgADP [28, 33-35] and MgAMPPNP [28, 36] and fluorescence of βY341W-mutant $\alpha_3\beta_3\gamma$-subcomplex of TF_1 was quenched by MgADP and MgATP [20, 30, 37], we have concluded that the relationship between the extent of nucleotide-induced quenching of the fluorescence of the engineered tryptophan residues and the extent of filling of three catalytic sites is not linear and that nucleotide binding to the third (lowest affinity) catalytic site contributes little if at all to the overall nucleotide-induced fluorescence quenching [32]. We have also suggested that MgADP binds to the third catalytic site of EcF_1 with nearly millimolar K_d.

In the present study, we tested this suggestion by investigating the MgADP concentration dependence of inactivation of the wild-type and βY331W-mutant EcF_1 by NBD-Cl. NBD-Cl inhibits F_1 [38] by modifying a specific tyrosine residue in β subunit (βY311 in MF_1 [39, 40] and βY307 in TF_1 [41, 42]). MF_1-βY311 and TF_1-βY307 are homologous to βY297 in EcF_1 [43], and mutation βY297F makes EcF_1 resistant to inactivation by NBD-Cl [44]. In the crystal structure of MF_1 inactivated by NBD-Cl, the Y331 residue of the β subunit that contains empty catalytic site (β_E) is modified [45]. Nucleotides [38, 46-49] and P_i [47-51] protect F_1 from inactivation by NBD-Cl.

The results presented here show that MgADP protects wild-type and βY331W-mutant EcF_1 by binding to a catalytic site with a K_d of 0.7 and ~10 mM, respectively, and support our conclusion [32] that nucleotide binding to the third (lowest affinity) catalytic site in βY331W-EcF_1 escapes detection by the fluorescence quenching method. The results also show that, during MgATP hydrolysis by EcF_1 in the absence of an activating anion, a catalytic site remains unoccupied at the nucleotide concentration as high as 10 mM and suggest that stimulation of MgATP hydrolysis by activating anions results from the anion binding to the third, unoccupied by nucleotide, catalytic site. These findings provide additional support for a bi-site mechanism of F_1 catalysis.

MATERIALS AND METHODS

Materials. ADP, ATP, NADH, Tris, triethanolamine, pyruvate kinase, and lyophilized lactate dehydrogenase were from Sigma (USA). Mops and potassium phosphoenolpyruvate were from Fluka (USA), H_2SeO_3 was from Aldrich (USA), dimethyl sulfoxide was from Baker (USA), and NBD-Cl was from Pierce (USA). Stock solutions of NBD-Cl (50 mM) were prepared in dry dimethyl sulfoxide and stored at −20°C protected from light. $KHSeO_3$ was prepared by titrating the H_2SeO_3 solution with KOH to pH 8.0. The pH of the stock solutions of ADP, ATP, and phosphoenolpyruvate was adjusted to 8.0 with triethanolamine.

Wild type EcF_1 and ε subunit [24] and βY331W-EcF_1 [32] were prepared as described previously. EcF_1 and βY331W-EcF_1 with the endogenous nucleotides bound at the catalytic sites removed as described previously [32] were used in the experiments. As discussed earlier [24], in order to avoid problems associated with ε subunit dissociation, all the experiments were carried out in the presence of saturating ε.

Inhibition of EcF_1 and βY331W-EcF_1 by NBD-Cl. To investigate the effect of MgADP on inhibition of the wild-type enzyme by NBD-Cl, 70 nM EcF_1 was incubated at room temperature (20-22°C) for 60 min in the medium containing 20 mM Mops/triethanolamine, pH 8.0, 2.2 mM $Mg(CH_3COO)_2$, 0.2 mM EDTA, 100 nM ε subunit, 0.1 mg/ml pyruvate kinase and MgADP (2-mM excess of Mg^{2+} in the medium ensured that practically all added nucleotide remained complexed with the cation). Then NBD-Cl was added from 50-mM stock solution to obtain the final concentration of 0.2 mM unless indicated otherwise, and ATPase activity was measured as described below using 40-μl aliquots of the reaction mixture after additional incubation for 0.5-38 min. When MgADP concentration was 10 and 20 mM, the EcF_1 concentration was increased to 280 nM and volume of the reaction mixture used to assay the ATPase activity was decreased to 10 μl. The effect of MgADP on inhibition of βY331W-EcF_1 by NBD-Cl was investigated under the same conditions but using the 85-nM enzyme at nucleotide concentrations lower than 10 mM and 0.42-μM enzyme when MgADP concentration was 10 and 20 mM.

To investigate inhibition by NBD-Cl during ATP hydrolysis, 60 nM EcF_1 was incubated at room temperature in medium containing 20 mM Mops/triethanolamine, pH 8.0, 2.2 mM $Mg(CH_3COO)_2$, 10 mM CH_3COOK, 0.2 mM EDTA, 1 mM phosphoenolpyruvate, 100 nM ε subunit, 0.1 mg/ml pyruvate kinase and MgATP in the absence and presence of 40 mM $KHSeO_3$ for 10 sec, then NBD-Cl was added to obtain the final concentration of 0.2 mM, and ATPase activity was measured as described below using 40-μl aliquots of the reaction mixture after additional incubation. In the absence of NBD-Cl, the enzyme activity was not affected when EcF_1 was incubated in the absence or presence of MgADP and MgATP and when βY331W-EcF_1 was incubated in the absence or presence of MgADP. The presence of selenite during 10-sec incubation increased EcF_1 activity by 25%.

The apparent pseudo-first-order rate constants of EcF_1 inhibition by NBD-Cl (k) were obtained by fitting the data to equation:

$$A_t = A_1 + A_2 e^{-kt}, \quad (1)$$

where A_t is ATPase activity of EcF_1 after incubation with NBD-Cl for time t.

ATPase activity assay. ATPase activity of EcF_1 and βY331W-EcF_1 was measured spectrophotometrically [52] at 340 nm at room temperature. The assay medium (final volume 1 ml) contained 20 mM Mops/Tris, pH 8.0, 0.2 mM EDTA, 3.2 mM $Mg(CH_3COO)_2$, 10 mM CH_3COOK, 1 mM phosphoenolpyruvate, 1 mM ATP, 0.3 mM NADH, 40 mM $KHSeO_3$, 100 nM ε subunit, 0.1 mg/ml pyruvate kinase, and 0.1 mg/ml lactate dehydrogenase. When concentration of carryover nucleotide (ADP and ATP) introduced to the assay medium with the sample exceeded 0.1 mM, the concentration of ATP in the assay medium was appropriately decreased in order to keep the total nucleotide concentration equal to 1 mM. ATPase activity of the samples containing NBD-Cl was calculated after correcting for a small rate of absorbance decrease due to presence of NBD-Cl determined using samples lacking EcF_1.

MgATP-concentration dependence of EcF_1 activity under conditions used for NBD-Cl-modification experiments was obtained using assay medium containing 20 mM Mops/triethanolamine, pH 8.0, 0.2 mM EDTA, 2.2 mM $Mg(CH_3COO)_2$, 10 mM CH_3COOK, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 100 nM ε subunit, 0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, and MgATP in the absence and presence of 40 mM $KHSeO_3$. The EcF_1 concentration was 12.2 and 5.7 nM in the absence and presence of selenite, respectively.

Protein assay. Protein was determined by a modified Lowry procedure [53] with BSA as a standard. A value of 380 kDa was used as the molecular mass of EcF_1 [54].

RESULTS

Effect of MgADP on inhibition of EcF_1 and βY331W-EcF_1 by NBD-Cl. The time course of EcF_1 inhibition by 0.2 mM NBD-Cl in the absence and presence of MgADP at three representative concentrations is shown in Fig. 1a. Both in the absence and presence of MgADP, the time course of inhibition is satisfactorily described by the exponential Eq. (1). NBD-Cl-induced inactivation of EcF_1 was completely reversed by incubation of the inactivated enzyme in the presence of 2 mM dithiothreitol for 10 min. This result is expected when the enzyme inhibition is due to modification of βY297 by NBD-Cl [38, 55, 56]. In the absence of MgADP, the time course of inhibition (curve *1*, Fig. 1a) corresponds well to the kinetics of EcF_1 inhibition by 0.1 mM NBD-Cl under the similar conditions reported by Ahmad and Senior [49] when the difference in the NBD-Cl concentration is taken into account. Figure 1b shows MgADP-concentration dependence of the rate constant k of EcF_1 inactivation by 0.2 mM NBD-Cl. It is seen that with increasing MgADP concentrations, the rate of EcF_1 inactivation initially increases reaching a maximum in the presence of 10 μM MgADP and then decreases. MgADP at high concentrations has been shown to protect the enzyme from inactivation by NBD-Cl [49]; however, the increased sensitivity of EcF_1 to NBD-Cl in the presence of micromolar MgADP has not been reported before.

The results presented in Fig. 2 show that the observed pseudo-first-order rate constant k for inhibition of EcF_1 by NBD-Cl is proportional to NBD-Cl concen-

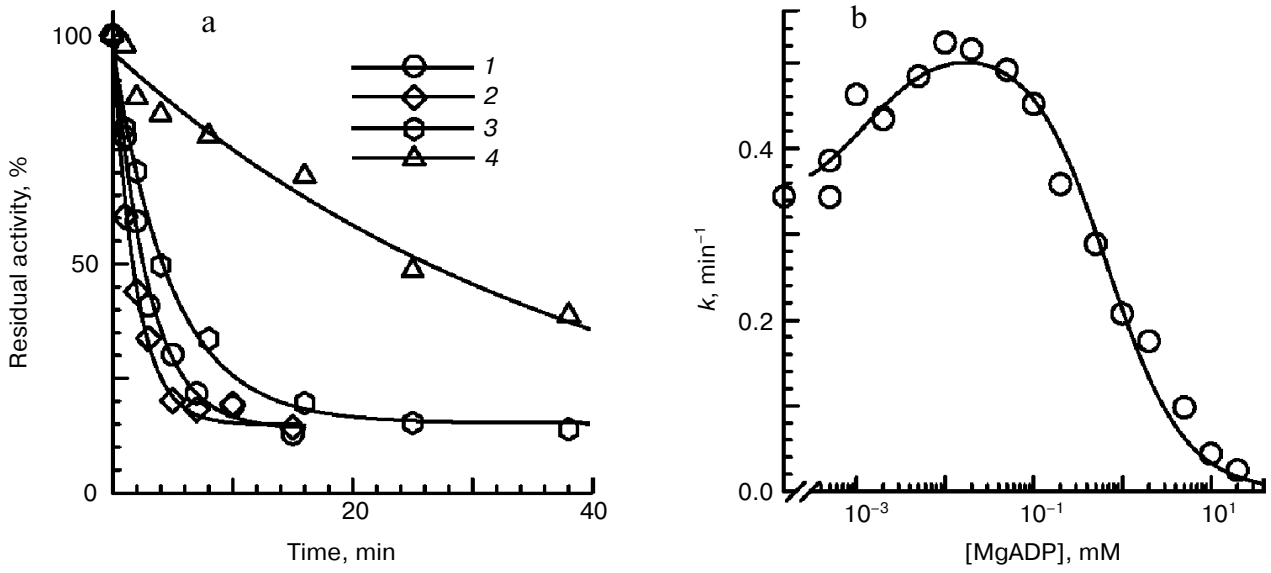

Fig. 1. Inhibition of EcF_1 by NBD-Cl. a) Time course of EcF_1 inhibition by 0.2 mM NBD-Cl in the absence (*1*) and presence of 0.01 (*2*), 1 (*3*), and 20 mM MgADP (*4*). Lines are drawn according to results of best fit of the corresponding data to Eq. (1). b) Effect of MgADP concentration on the rate constant k of EcF_1 inhibition by 0.2 mM NBD-Cl.

tration both at low and high MgADP concentrations. Values of k for inhibition of EcF_1 by NBD-Cl in the absence of MgADP were also reported to be proportional to NBD-Cl concentration [49]. Thus, the results of Fig. 2 mean that the rate of EcF_1 inhibition by 0.2 mM NBD-Cl is limited by the rate of NBD-Cl binding to EcF_1. This circumstance allows using the MgADP concentration dependence of k (Fig. 1b) to obtain the K_d values for MgADP binding to catalytic sites of EcF_1 that affects the enzyme inactivation by NBD-Cl.

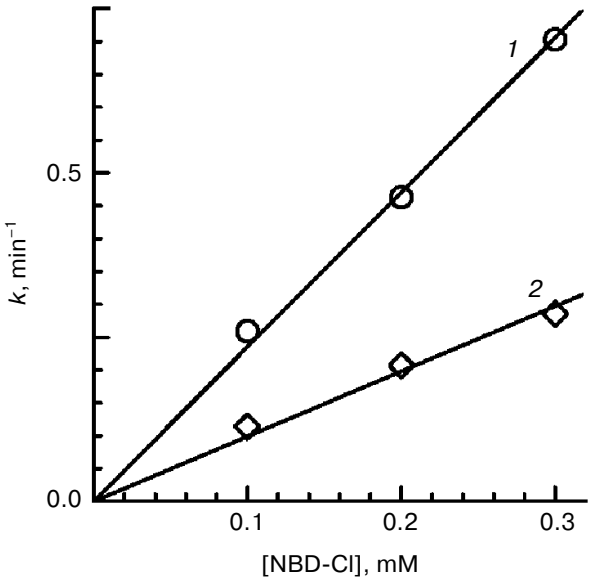

Fig. 2. Effect of NBD-Cl concentration on the rate constant k of EcF_1 inhibition in the presence of 1 μM (*1*) and 1 mM (*2*) MgADP. Rate constants k were obtained as described in "Materials and Methods" using indicated concentrations of NBD-Cl. Slopes of the lines obtained using the linear regression analysis of the data are equal to $(2.4 \pm 0.05)\cdot10^3$ (*1*) and $(1 \pm 0.04)\cdot10^3$ $M^{-1}\cdot min^{-1}$ (*2*).

The line in Fig. 1b represents the results of the best fit of the data to equation:

$$k = (k_0 + k_2 S/K_{d2})/[1 + S/K_{d2} + S^2/(K_{d2}\cdot K_{d3})], \qquad (2)$$

where k_0 is the rate constant of EcF_1 inhibition by 0.2 mM NBD-Cl in the absence of nucleotide, k_2 is the rate constant of the enzyme inhibition after MgADP binding at a catalytic site with a dissociation constant K_{d2}, K_{d3} is the dissociation constant for a catalytic site where MgADP binding completely protects the enzyme from inactivation by NBD-Cl, and S is the concentration of MgADP. Equation (2) is based on a model that assumes that MgADP binding at two catalytic sites modulates reactivity of EcF_1 toward NBD-Cl. In this model, MgADP binding at the catalytic site with higher affinity increases the reactivity, and the nucleotide binding at the lower affinity catalytic site results in complete protection of EcF_1 from inactivation by NBD-Cl. The best-fit values of k_0 and k_2 are equal to 0.33 ± 0.03 and 0.53 ± 0.02 min^{-1}, respectively, and the best fit values of K_{d2} and K_{d3} are equal to 1.3 ± 0.7 μM and 0.7 ± 0.1 mM, respectively. The K_{d3} value for MgADP of 0.7 mM obtained from the data shown in Fig. 1b is in good agreement with the K_d value of about 0.5 mM for the lowest affinity catalytic site of EcF_1 we have estimated recently [32] based on the results presented by Ahmad and Senior [49].

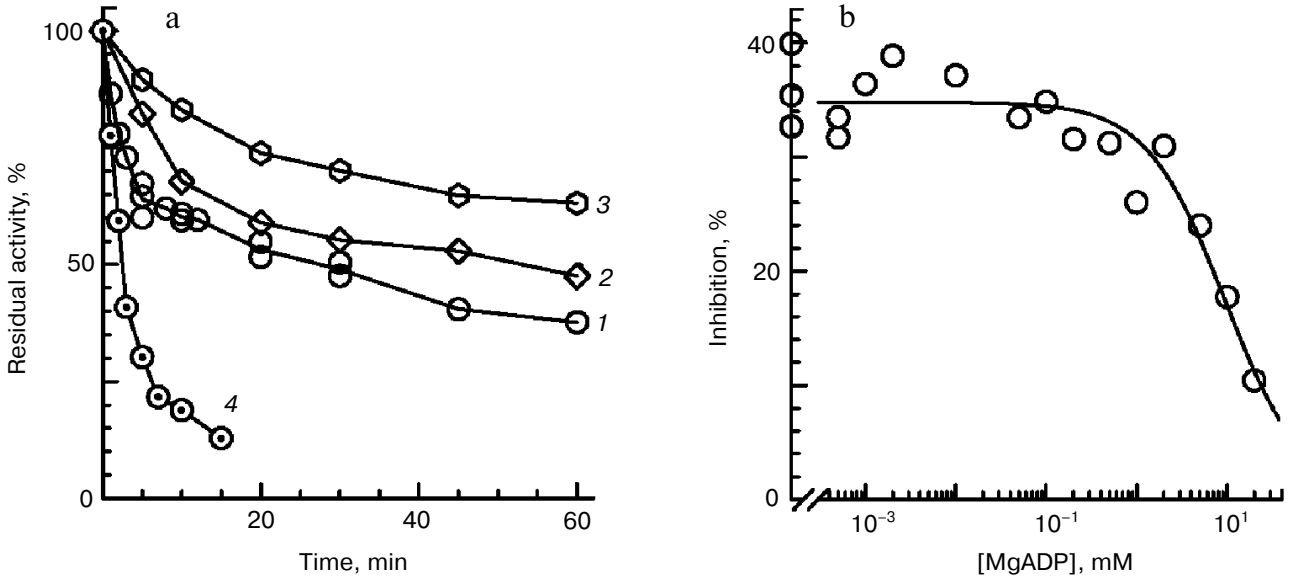

Fig. 3. Inhibition of βY331W-EcF_1 by NBD-Cl. a) Time course of βY331W-EcF_1 inhibition by 0.2 mM NBD-Cl in the absence (*1*) and presence of 10 (*2*) and 20 mM (*3*) MgADP. 100% activity for βY331W-EcF_1 corresponds to 9.2 sec^{-1}. For comparison, curve *4* shows the time course of wild-type EcF_1 inhibition by 0.2 mM NBD-Cl in the absence of MgADP. b) Effect of MgADP concentration on βY331W-EcF_1 inhibition by NBD-Cl. βY331W-EcF_1 was preincubated with and without MgADP for 1 h and assayed after additional incubation for 5 min in the presence of 0.2 mM NBD-Cl as described in "Materials and Methods". The line represents the best fit of the data to the hyperbolic equation $I = I_0/(1 + S/K_d)$, where I_0 is the inhibition in the absence of MgADP, S is the concentration of MgADP, and K_d is the dissociation constant for MgADP. The best fit values of I_0 and K_d are equal to 35 ± 1% and 10 ± 2 mM, respectively.

Figure 3a shows time course of βY331W-EcF_1 inhibition by 0.2 mM NBD-Cl in the absence and presence of MgADP. For comparison, Fig. 3a also shows the course of wild-type EcF_1 inhibition by 0.2 mM NBD-Cl in the absence of MgADP. It is seen that kinetics of βY331W-EcF_1 inhibition is not monoexponential. For this reason, to evaluate the effect of MgADP on βY331W-EcF_1 inhibition by NBD-Cl we plotted in Fig. 3b the extent of inhibition observed after 5-min incubation with 0.2 mM NBD-Cl versus the MgADP concentration. The results show that MgADP at concentrations up to about 1 mM does not affect significantly inhibition of βY331W-EcF_1 by NBD-Cl, but at higher concentrations slows down NBD-Cl-induced inhibition of the enzyme. Fitting the results shown in Fig. 3b to a hyperbolic equation yielded an apparent K_d value for MgADP of 10 ± 2 mM.

Inhibition of EcF_1 by NBD-Cl during ATP hydrolysis. Within the framework of a bi-site catalytic mechanism, the substrate modulation of multi-site activity of F_1 is due to the substrate binding to the second catalytic site. On the other hand, in tri-site models of the F_1 catalysis, filling the second catalytic site produces little or no acceleration of catalysis and the substrate binding to the third site is required for rapid enzyme turnover [30, 31, 57-59]. According to tri-site models, all three catalytic sites should be filled by substrate and intermediately bound products during MgATP hydrolysis at saturating substrate concentrations, while one catalytic site can remain unoccupied under these conditions according to a bi-site model. Therefore one can expect that EcF_1 would remain sensitive to inhibition by NBD-Cl in the presence of saturating MgATP concentrations if the enzyme operates according to a bi-site model. To test this possibility, we investigated MgATP effect on EcF_1 inactivation by 0.2 mM NBD-Cl. To avoid interference from product MgADP rebinding at the catalytic sites of EcF_1, the experiments were conducted in the presence of pyruvate kinase and 1 mM phosphoenolpyruvate using a sufficiently low EcF_1 concentration to prevent the exhaustion of the phosphoenolpyruvate. That pyruvate kinase remained active during EcF_1 incubation with NBD-Cl had been established in the control experiments, in which incubation of pyruvate kinase in the presence of 0.2 mM NBD-Cl for up to 1 h inhibited this enzyme by less than 15%.

Curve *1* in Fig. 4a demonstrates the effect of MgATP on the apparent rate constant k of EcF_1 inhibition by 0.2 mM NBD-Cl. It is seen that MgATP at concentrations up to 2 μM does not affect inhibition of EcF_1 by NBD-Cl. Further increase in MgATP concentration results in a decrease in k values. This decrease in the k values levels off at about 0.1 min^{-1} in the presence of 0.2 mM MgATP, and subsequent increase in MgATP concentration up to 10 mM does not affect the k value. Fitting the data to a hyperbolic equation resulted in the best fit values for k of 0.34 ± 0.02 and 0.1 ± 0.01 min^{-1} in the absence of MgATP and in the presence of the nucleotide at satu-

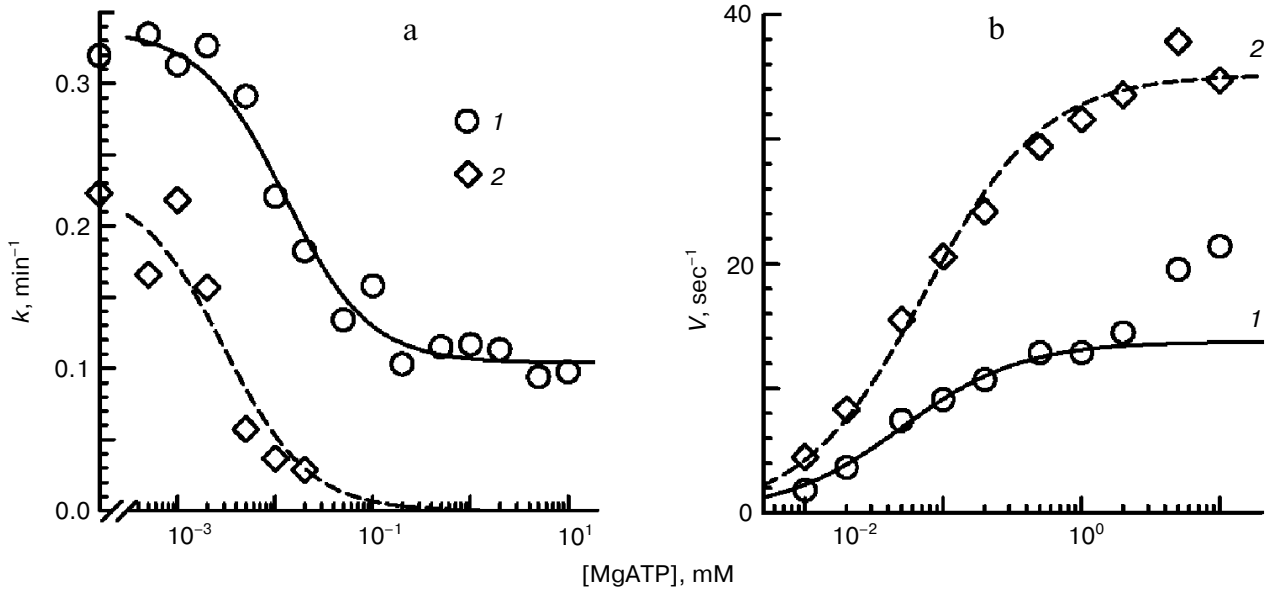

Fig. 4. Effect of MgATP concentration on the rate constant k of EcF_1 inhibition by 0.2 mM NBD-Cl (a) and the enzyme activity (b) in the absence (*1*) and presence (*2*) of 40 mM selenite. a) Rate constants k were obtained as described in "Materials and Methods". Lines represent the best fit of the data to a hyperbolic equation $k = k_1 + k_2/(1 + S/K_{1/2})$, where S is the MgATP concentration, $k_1 + k_2$ and k_1 are the rate constants of inhibition in the absence of MgATP and at the infinite MgATP concentration, respectively, and $K_{1/2}$ is the concentration of MgATP required for half-maximal decrease of k. When fitting the data obtained in the presence of selenite, k_1 was assumed to be equal to zero. The best fit values of k_1, k_2, and $K_{1/2}$ for the data obtained in the absence of selenite (*1*) are 0.10 ± 0.01 min^{-1}, 0.24 ± 0.01 min^{-1}, and 12.5 ± 3 μM, respectively, and the best fit values of k_2 and $K_{1/2}$ for the data obtained in the presence of selenite (*2*) are 0.23 ± 0.02 min^{-1} and 3 ± 1 μM, respectively. b) Lines represent the best fit of the data to the Michaelis–Menten equation. Only activity values obtained at MgATP concentrations ≤1 mM were used to fit the data obtained in the absence of selenite (*1*). The best fit values of K_m and V_{max} are equal to 51 ± 5 μM and 13.7 ± 0.4 sec^{-1}, respectively, in the absence of selenite (*1*), and to 73 ± 8 μM and 35 ± 0.8 sec^{-1}, respectively, in the presence of selenite (*2*).

rating concentration, respectively. The best fit (Fig. 4a, curve *1*) also yielded a value for MgATP concentration inducing a half-maximal change in k ($K_{1/2}$) of 12.5 ± 3 μM. The results (curve *1*, Fig. 4a) show that Y297 in apparently one β subunit of EcF_1 remains available for modification by NBD-Cl during hydrolysis of MgATP at concentrations as high as 10 mM.

Circles in Fig. 4b show MgATP-concentration dependence of EcF_1 activity under conditions used in the experiments represented by curve *1* in Fig. 4a. It is apparent that this dependence does not exhibit simple Michaelis–Menten behavior. However, in the range of MgATP concentrations ≤1 mM, the substrate-concentration dependence of EcF_1-ATPase activity fits well to the Michaelis–Menten equation with the best fit K_m and V_{max} values of 51 ± 5 μM and 13.7 ± 0.4 sec^{-1}, respectively (Fig. 4b, solid line *1*). Formation of an inactive $MgADP{\cdot}F_1$ complex with the inhibitory MgADP bound at one of the catalytic sites [15] has been shown to be responsible for deviations from the Michaelis–Menten behavior during ATP hydrolysis by F_1 [18, 21, 22, 58] and for well known inhibition of EcF_1-ATPase activity by free Mg^{2+} [60]. The deviation from Michaelis–Menten behavior in MgATP-concentration dependence of EcF_1 activity in the presence of free Mg^{2+} (Fig. 4b, circles) is apparently also due to the presence of an inactive EcF_1 species with the inhibitory MgADP bound at one of the catalytic sites, and the increase in EcF_1 activity at MgATP concentrations >1 mM is most likely the result of a decrease in the fraction of the inactive enzyme due to low-affinity MgATP binding. Nucleotide binding to noncatalytic sites has been shown to affect the dynamic steady state between MgADP-inhibited and active F_1 forms during ATP hydrolysis [61-66]. However, even if the increase of ATPase activity of EcF_1 at MgATP concentration >1 mM (Fig. 4b, circles) is due to nucleotide binding at a catalytic site, the results represented by curve *1* in Fig. 4a show that one catalytic site remains mostly unoccupied even in the presence of 10 mM MgATP.

Selenite, one of the activating anions [16], stimulates MgATPase activity of EcF_1 ([24] and Fig. 4b, diamonds). This stimulation is likely due to a decrease in a relative content of MgADP-inhibited EcF_1 because activating anions have been shown to accelerate reactivation of MgADP-inhibited enzyme [67, 68]. In the presence of selenite, the substrate-concentration dependence of MgATPase activity of EcF_1 does not deviate significantly from a hyperbola with K_m and V_{max} values of 73 ± 8 μM and 35 ± 0.8 sec^{-1}, respectively (Fig. 4b, curve *2*). In the absence of added MgATP, selenite slows down inhibition of EcF_1 by 0.2 mM NBD-Cl (Fig. 4a). Selenite also increases efficiency of MgATP in protecting EcF_1 from

inactivation by NBD-Cl (Fig. 4a, curve *2*). Fitting the data obtained in the presence of selenite (diamonds in Fig. 4a) to a hyperbolic equation yielded a value of $K_{1/2}$ for MgATP of 3 ± 1 μM (Fig. 4a, curve *2*). Selenite-induced decrease in *k* values in the absence of MgATP (~0.1 min^{-1}) is equal to values of *k* obtained in the absence of selenite at MgATP concentrations ≥0.2 mM suggesting that selenite can slow down the NBD-Cl-induced inactivation of EcF_1 by binding at the catalytic site that remains unoccupied during enzyme turnover at saturating substrate concentrations.

DISCUSSION

The results presented in this study (Fig. 1b) show that inactivation of EcF_1 by NBD-Cl is affected by MgADP binding at two catalytic sites. It is likely that the MgADP-induced protection from inhibition by NBD-Cl with a K_d for the nucleotide of 0.7 mM is due to MgADP binding to the catalytic site with the lowest affinity to the nucleotide, namely, the third catalytic site, as it was suggested by Ahmad and Senior [49]. The K_d value for MgADP obtained in the present study (0.7 mM) is very close to the value of about 0.5 mM that was estimated earlier [32] using the data presented by Ahmad and Senior [49]. The K_{d3} value of 0.7 mM for MgADP is also consistent with the results of Pagan and Senior [69], who using the column-centrifugation method [70] detected about 2.5 moles of nucleotide/mole of EcF_1 bound to the catalytic sites after incubation in the presence of 1.25 mM MgADP. However, it is not clear whether the MgADP-induced increase in sensitivity of EcF_1 toward inhibition by NBD-Cl with a K_d of 1.3 μM at pH 8.0 (Fig. 1b) is due to the nucleotide binding to the first or the second catalytic site. The K_d values for MgADP binding at the first catalytic site of EcF_1 were estimated from the kinetics of the nucleotide binding and dissociation to be 8.8 and 1.7 μM [71] at pH 7.5 and 8.5, respectively. Two binding sites with a K_d of 3 μM for MgADP were detected in EcF_1 [3] when nucleotide binding to catalytic sites was measured using the column-centrifugation method [70]. It appears however that affinity of the first catalytic site to MgADP reported earlier [3, 71] might have been underestimated due to presence of sulfate in the incubation media. Sulfate has been shown to significantly increase the K_d value for MgADP binding at the first catalytic site of βY331W-EcF_1 [32]. For this reason, we believe that MgADP binding at the first catalytic site does not significantly affect EcF_1 inhibition by NBD-Cl, and it is MgADP binding at the second catalytic site with a K_d of 1.3 μM that increases sensitivity of EcF_1 to inhibition by NBD-Cl (Fig. 1a).

Under identical conditions, βY331W-EcF_1 is less susceptible to inhibition by NBD-Cl than the wild-type enzyme (Fig. 3a). In this regard, it should be mentioned that double mutant enzymes containing βY331W-mutation and additionally one of the mutations βR246Q, βR246K, or βR246A [49], βN243D [72], βR246A, βR243R, or αF291R [73], αF291D or αF291E [74] are also less susceptible to inhibition by NBD-Cl when compared to the wild-type EcF_1. Unlike the wild-type enzyme, βY331W-EcF_1 does not exhibit increased sensitivity to NBD-Cl in the presence of micromolar MgADP (Fig. 3b). However, βY331W-EcF_1 similarly to the wild-type enzyme is protected from inactivation by NBD-Cl when MgADP binds to a low-affinity (K_d about 10 mM) catalytic site (Fig. 3b). When βY331W-EcF_1 [28, 33, 35, 75] and ε-depleted βY331W-EcF_1 [32, 76] were titrated with MgADP, the extent of the nucleotide-induced fluorescence quenching exhibited saturation at about 0.1 mM MgADP with little or no significant increase at higher nucleotide concentrations. Therefore, the fact that MgADP protects βY331W-EcF_1 from inactivation by NBD-Cl by binding to a catalytic site with a K_d of about 10 mM (Fig. 3b) lends additional support to our conclusion [32] that the relationship between occupancy of the three catalytic sites and the nucleotide-induced fluorescence quenching in βY331W-EcF_1 is not linear and that nucleotide binding to the third catalytic site of βY331W-EcF_1 contributes little if at all to the overall nucleotide-induced fluorescence quenching. This lack of linearity significantly complicates analysis of data obtained using the fluorescence-quenching approach, and might have contributed to mistaken interpretation of the data in favor of a tri-site mechanism.

The result that MgATP at concentration as high as 10 mM only partially protects EcF_1 from inactivation by NBD-Cl (Fig. 4a, curve *1*) supports our conclusion [24] that a bi-site mechanism is responsible for catalysis of MgATP hydrolysis by EcF_1. Obviously, one catalytic site remains mostly unoccupied by nucleotide during millimolar MgATP hydrolysis by EcF_1 in the absence of an activating anion. The effect of selenite on MgATP-concentration dependence of EcF_1 inhibition by NBD-Cl (Fig. 4a, curve *2*) gives evidence for the anion binding to this unoccupied catalytic site. Stimulating effect of activating anions on ATP hydrolysis by F_1 was originally proposed to be due to their competing with MgATP for binding to noncatalytic nucleotide-binding sites [77] that were considered to be specific for adenine nucleotides [78]. However, the results of the present study suggest the hypothesis that stimulation by selenite and other activating anions of MgATP hydrolysis by MF_1 [16] and EcF_1 [24, 79] is due to anion binding to the catalytic site that remains free of nucleotide at saturating substrate concentrations. In this regard, it should be mentioned that an electron density consistent with bound sulfate anion is found at different positions in the free of nucleotide catalytic site in the crystal structures of MF_1 [80], dicyclohexylcarbodiimide-inhibited MF_1 [81], and yeast MF_1 [82].

Both in the absence and presence of selenite, the concentration of MgATP required for half-maximal decrease of the rate constant k of EcF_1 inhibition by NBD-Cl ($K_{1/2}$, Fig. 4a) is smaller than the corresponding K_m value (Fig. 4b). Obviously, the increase of proportion of the enzyme molecules with two catalytic site occupied that is responsible for substrate-concentration modulation of EcF_1 activity (Fig. 4b) cannot explain the decrease of k values that takes place mostly in the range of MgATP concentrations < K_m (Fig. 4a). Indeed, the catalytic site occupancy exhibited an increase of only 0.2-0.3 mole of nucleotide/mole of EcF_1 when MgATP concentration was increased from 1 to 20 μM both in the absence and presence of selenite [24]. We propose that MgATP-induced decrease of EcF_1 sensitivity to NBD-Cl at the substrate concentrations < K_m (Fig. 4a) is due to an increased abundance of the enzyme species with one catalytic site occupied by MgATP. During steady-state MgATP hydrolysis by EcF_1 when the substrate concentration << K_m, the enzyme exists practically only in a state with only one catalytic site occupied either by MgATP or by MgADP with or without P_i. The latter species may include also the MgADP-inhibited enzyme form that is more abundant in the absence of selenite. In the active fraction of EcF_1, the relative abundance of the enzyme species having at a single catalytic site either ATP or ADP with or without P_i is determined mainly by the rates of interconversion ATP ↔ ADP + P_i at a single catalytic site and by MgATP concentration. We propose that when MgATP is bound at the catalytic site of one β subunit, Y297 residues in one or both β subunits with unoccupied catalytic sites are significantly less reactive toward NBD-Cl than when MgADP is bound at a single catalytic site with or without P_i. With the increase in MgATP concentration, the rate of the nucleotide binding to the second catalytic site is increased, which results in the increased abundance of the enzyme species with MgATP bound at a single catalytic site and, as a consequence, in the decreased reactivity toward NBD-Cl. This kinetic mechanism explains why $K_{1/2}$ values for MgATP (Fig. 4a) are lower than the corresponding K_m values (Fig. 4b) and is similar to the mechanism proposed to explain modulation of the intermediate H_2O/P_i-oxygen exchange by the substrate concentration during MgATP hydrolysis by MF_1 [13, 83].

We thank Dr. Paul D. Boyer for many discussions and advice.

REFERENCES

1. Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994) *Nature*, **370**, 621-628.
2. Cross, R. L., and Nalin, C. M. (1982) *J. Biol. Chem.*, **257**, 2874-2881.
3. Wise, J. G., Duncan, T. M., Latchney, L. R., Cox, D. N., and Senior, A. E. (1983) *Biochem. J.*, **215**, 343-350.
4. Girault, G., Berger, G., Galmiche, J.-M., and Andre, F. (1988) *J. Biol. Chem.*, **263**, 14690-14695.
5. Boyer, P. D., Cross, R. L., and Momsen, W. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 2837-2839.
6. Kayalar, C., Rosing, J., and Boyer, P. D. (1977) *J. Biol. Chem.*, **252**, 2486-2491.
7. Boyer, P. D., and Kohlbrenner, W. E. (1981) in *Energy Coupling in Photosynthesis* (Selman, B. R., and Selman-Reiner, S., eds.) Elsevier North Holland, New York, pp. 231-240.
8. Kinosita, K., Jr., Yasuda, R., Noji, H., and Adachi, K. (2000) *Philos. Trans. R. Soc. Lond., B, Biol. Sci.*, **355**, 473-489.
9. Stock, D., Gibbons, C., Arechaga, I., Leslie, A. G. W., and Walker, J. E. (2000) *Curr. Opin. Struct. Biol.*, **10**, 672-679.
10. Boyer, P. D. (1993) *Biochim. Biophys. Acta*, **1140**, 215-250.
11. Boyer, P. D. (2001) *Biochemistry (Moscow)*, **66**, 1058-1066.
12. Grubmeyer, C., Cross, R. L., and Penefsky, H. S. (1982) *J. Biol. Chem.*, **257**, 12092-12100.
13. Cross, R. L., Grubmeyer, C., and Penefsky, H. S. (1982) *J. Biol. Chem.*, **257**, 12101-12105.
14. Duncan, T. M., and Senior, A. E. (1985) *J. Biol. Chem.*, **260**, 4901-4907.
15. Milgrom, Y. M., and Boyer, P. D. (1990) *Biochim. Biophys. Acta*, **1020**, 43-48.
16. Ebel, R. E., and Lardy, H. A. (1975) *J. Biol. Chem.*, **250**, 191-196.
17. Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., and Vinogradov, A. D. (1982) *Biochem. J.*, **202**, 9-14.
18. Vulfson, E. N., Drobinskaya, I. E., Kozlov, I. A., and Murataliev, M. B. (1986) *Biol. Membr. (Moscow)*, **3**, 339-351.
19. Weber, J., and Senior, A. E. (1997) *Biochim. Biophys. Acta*, **1319**, 19-58.
20. Ren, H., and Allison, W. S. (2000) *J. Biol. Chem.*, **275**, 10057-10063.
21. Yasuda, R., Noji, H., Yosida, M., Kinosita, K., Jr., and Itoh, H. (2001) *Nature*, **410**, 898-904.
22. Sakaki, N., Shimo-Kon, R., Adachi, K., Itoh, H., Furuike, S., Muneyuki, E., Yoshida, M., and Kinosita, K., Jr. (2005) *Biophys. J.*, **88**, 2047-2056.
23. Milgrom, Y. M., Murataliev, M. B., and Boyer, P. D. (1998) *Biochem. J.*, **330**, 1037-1043.
24. Bulygin, V. V., and Milgrom, Y. M. (2009) *Biochim. Biophys. Acta*, **1787**, 1016-1023.
25. Zhou, J.-M., and Boyer, P. D. (1993) *J. Biol. Chem.*, **268**, 1531-1538.
26. Murataliev, M. B., and Boyer, P. D. (1994) *J. Biol. Chem.*, **269**, 15431-15439.
27. Milgrom, Y. M., and Cross, R. L. (2005) *Proc. Natl. Acad. Sci. USA*, **102**, 13831-13836.
28. Weber, J., Wilke-Mounts, S., Lee, R. S.-F., Grell, E., and Senior, A. E. (1993) *J. Biol. Chem.*, **268**, 20126-20133.
29. Dou, C., Fortes, P. A. G., and Allison, W. S. (1998) *Biochemistry*, **37**, 16757-16764.
30. Ono, S., Hara, K. Y., Hirao, J., Matsui, T., Noji, H., Yoshida, M., and Muneyuki, E. (2003) *Biochim. Biophys. Acta*, **1607**, 35-44.
31. Corvest, V., Sigalat, C., Venard, R., Falson, P., Mueller, D. M., and Haraux, F. (2005) *J. Biol. Chem.*, **280**, 9927-9936.

32. Bulygin, V. V., and Milgrom, Y. M. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 4327-4331.
33. Lobau, S., Weber, J., Wilke-Mounts, S., and Senior, A. E. (1997) *J. Biol. Chem.*, **272**, 3648-3656.
34. Weber, J., and Senior, A. E. (1998) *J. Biol. Chem.*, **273**, 33210-33215.
35. Nadanaciva, S., Weber, J., and Senior, A. E. (1999) *J. Biol. Chem.*, **274**, 7052-7058.
36. Weber, J., and Senior, A. E. (1995) *J. Biol. Chem.*, **270**, 12653-12658.
37. Bandyopadhyay, S., Valder, C. R., Huynh, H. G., Ren, H., and Allison, W. S. (2002) *Biochemistry*, **41**, 14421-14429.
38. Ferguson, S. J., Lloyd, W. J., Lyons, M. H., and Radda, G. K. (1975) *Eur. J. Biochem.*, **54**, 117-126.
39. Andrews, W. W., Hill, F. C., and Allison, W. S. (1984) *J. Biol. Chem.*, **259**, 8219-8225.
40. Sutton, R., and Ferguson, S. J. (1985) *Eur. J. Biochem.*, **148**, 551-554.
41. Verburg, J. G., Yoshida, M., and Allison, W. S. (1986) *Arch. Biochem. Biophys.*, **245**, 8-13.
42. Yoshida, M., and Allison, W. S. (1990) *J. Biol. Chem.*, **265**, 2483-2487.
43. Senior, A. E. (1990) *Annu. Rev. Biophys. Biophys. Chem.*, **19**, 7-41.
44. Parsonage, D., Wilke-Mounts, S., and Senior, A. E. (1987) *J. Biol. Chem.*, **262**, 8022-8026.
45. Orriss, G. L., Leslie, A. G. W., Braig, K., and Walker, J. E. (1998) *Structure (Lond.)*, **6**, 831-837.
46. Steinmeier, R. C., and Wang, J. H. (1979) *Biochemistry*, **18**, 11-18.
47. Ting, L. P., and Wang, J. H. (1980) *J. Bioenerg. Biomembr.*, **12**, 79-93.
48. Cortez, N., Lucero, H. A., and Vallejos, R. H. (1983) *Biochim. Biophys. Acta*, **724**, 396-403.
49. Ahmad, Z., and Senior, A. E. (2004) *J. Biol. Chem.*, **279**, 31505-31513.
50. Ting, L. P., and Wang, J. H. (1980) *Biochemistry*, **19**, 5665-5670.
51. Perez, J. A., Greenfield, A. J., Sutton, R., and Ferguson, S. J. (1986) *FEBS Lett.*, **198**, 113-118.
52. Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960) *J. Biol. Chem.*, **235**, 3322-3329.
53. Peterson, G. L. (1977) *Anal. Biochem.*, **83**, 346-356.
54. Walker, J. E., Saraste, M., and Gay, N. J. (1984) *Biochim. Biophys. Acta*, **768**, 164-200.
55. Ferguson, S. J., Lloyd, W. J., and Radda, G. K. (1975) *Eur. J. Biochem.*, **54**, 127-133.
56. Lunardi, J., Satre, M., Bof, M., and Vignais, P. V. (1979) *Biochemistry*, **18**, 5310-5321.
57. Weber, J., and Senior, A. E. (2001) *J. Biol. Chem.*, **276**, 35422-35428.
58. Ren, H., Bandyopadhyay, S., and Allison, W. S. (2006) *Biochemistry*, **45**, 6222-6230.
59. Ariga, T., Muneyuki, E., and Yoshida, M. (2007) *Nat. Struct. Mol. Biol.*, **14**, 841-846.
60. Hyndman, D. J., Milgrom, Y. M., Bramhall, E. A., and Cross, R. L. (1994) *J. Biol. Chem.*, **269**, 28871-28877.
61. Murataliev, M. B. (1992) *Biochemistry*, **31**, 12885-12892.
62. Jault, J.-M., and Allison, W. A. (1993) *J. Biol. Chem.*, **268**, 1558-1566.
63. Milgrom, Y. M., and Cross, R. L. (1993) *J. Biol. Chem.*, **268**, 23179-23185.
64. Paik, S. R., Jault, J.-M., and Allison, W. S. (1994) *Biochemistry*, **33**, 126-133.
65. Jault, J.-M., Matsui, T., Jault, F. M., Kaibara, C., Muneyuki, E., Yoshida, M., Kagawa, Y., and Allison, W. S. (1995) *Biochemistry*, **34**, 16412-16418.
66. Matsui, T., Muneyuki, E., Honda, M., Allison, W. S., Dou, C., and Yoshida, M. (1997) *J. Biol. Chem.*, **272**, 8215-8221.
67. Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., and Vinogradov, A. D. (1982) *Biochem. J.*, **202**, 15-23.
68. Kalashnikova, T. Y., Milgrom, Y. M., and Murataliev, M. B. (1988) *Eur. J. Biochem.*, **177**, 213-218.
69. Pagan, J., and Senior, A. E. (1990) *FEBS Lett.*, **273**, 147-149.
70. Penefsky, H. S. (1977) *J. Biol. Chem.*, **252**, 2891-2899.
71. Al-Shawi, M. K., and Senior, A. E. (1992) *Biochemistry*, **31**, 878-885.
72. Ahmad, Z., and Senior, A. E. (2004) *J. Biol. Chem.*, **279**, 46057-46064.
73. Ahmad, Z., and Senior, A. E. (2005) *J. Biol. Chem.*, **280**, 27981-27989.
74. Brudecki, L. E., Grindstaff, J. J., and Ahmad, Z. (2008) *Arch. Biochem. Biophys.*, **471**, 168-175.
75. Mao, H. Z., Gray, W. D., and Weber, J. (2006) *FEBS Lett.*, **580**, 4131-4135.
76. Weber, J., Dunn, S. D., and Senior, A. E. (1999) *J. Biol. Chem.*, **274**, 19124-19128.
77. Recktenwald, D., and Hess, B. (1977) *FEBS Lett.*, **76**, 25-28.
78. Schuster, S. M., Ebel, R. E., and Lardy, H. A. (1975) *J. Biol. Chem.*, **250**, 7848-7853.
79. Dunn, S. D., Zadorozny, V. D., Tozer, R. G., and Orr, L. E. (1987) *Biochemistry*, **26**, 4488-4493.
80. Menz, R. I., Leslie, A. G. W., and Walker, J. E. (2001) *FEBS Lett.*, **494**, 11-14.
81. Gibbons, C., Montgomery, M. G., Leslie, A. G. W., and Walker, J. E. (2000) *Nat. Struct. Biol.*, **7**, 1055-1061.
82. Kabaleeswaran, V., Puri, N., Walker, J. E., Leslie, A. G. W., and Mueller, D. M. (2006) *EMBO J.*, **25**, 5433-5442.
83. Choate, G. L., Hutton, R. L., and Boyer, P. D. (1979) *J. Biol. Chem.*, **254**, 286-290.