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MgATP-concentration dependence of protection of yeast vacuolar V-ATPase from inactivation by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole supports a bi-site catalytic mechanism of ATP hydrolysis

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ABSTRACT

Catalytic site occupancy of the yeast vacuolar V-ATPase during ATP hydrolysis in the presence of an ATP-regenerating system was probed using sensitivity of the enzyme to inhibition by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The results show that, regardless of the presence or absence of the proton-motive force across the vacuolar membrane, saturation of V-ATPase activity at increasing MgATP concentrations is accompanied by only partial protection of the enzyme from inhibition by NBD-Cl. Both in the presence and absence of an uncoupler, complete protection of V-ATPase from inhibition by NBD-Cl requires MgATP concentrations that are significantly higher than those expected from the K_m values for MgATP. The results are inconsistent with a tri-site model and support a bi-site model for a mechanism of ATP hydrolysis by V-ATPase.

1. Introduction

Vacuolar type (V-type) H^+-ATPases are proton pumps responsible in eukaryotic cells for acidification of the various intracellular and extracellular compartments and are important for many cellular functions (see [1–5] for recent reviews). V-ATPase is a large multisubunit complex that is structurally and mechanistically related to F_oF_1-ATPase (ATP synthase). Two main domains are functionally and structurally recognized in V-ATPase – a membrane-imbedded domain called V_o (by analogy to factor F_o of the F_oF_1-ATPase) responsible for the transmembrane transport of protons, and a peripheral domain called V_1 responsible for ATP hydrolysis. The V_o and V_1 domains are composed of six (*a*, *c*, *c*′, *c*″, *d*, *e*) and eight (A, B, C, D, E, F, G and H) different types of subunits, respectively, and are connected by one centrally located stalk and by a few peripheral stalks. It is generally accepted [1–5] that ATP hydrolysis is coupled to a proton transport in V-ATPase by a rotary mechanism similar to a rotary binding change mechanism originally proposed by Boyer and colleagues for F_oF_1-ATPase [6–8]. It is thought that ATP-dependent proton translocation by V-ATPase involves a rotation of the centrally located stalk composed of D, F, and *d* subunits and a ring of *c*, *c*′, and *c*″ subunits (rotor). This rotation is assumed to be driven by conformational changes of the three catalytic A subunits of V_1 that are induced by ATP binding to and ADP and P_i dissociation from the three catalytic sites. ATP-driven rotation has been directly observed both in F_1 [9] and V-ATPase [10].

According to the binding change mechanism, during ATP hydrolysis each of the three catalytic sites sequentially progresses through identical set of states in a series of tightly coordinated conformational transitions [11]. Cooperativity among catalytic sites was predicted to result in a slow enzyme turnover when a substrate was bound at only one catalytic site and in an acceleration of turnover upon substrate binding at additional catalytic site(s) [7]. Such enzyme behavior has been directly observed first with MF_1 [12,13] and subsequently with V-ATPase [14] and explains the substrate-concentration dependence of the intermediate H_2O/P_i-oxygen exchange during ATP hydrolysis by MF_1 [15] and V-ATPase [16]. The slow enzyme turnover with only one catalytic site occupied has been named uni-site catalysis [12], and the cooperative modes of the enzyme turnover have been named bi-site and tri-site catalysis in accordance with the presumed extent of occupancy of the three catalytic sites [13] and are often referred to as multi-site catalysis. The issue of relative contribution of bi-site and tri-site catalysis to enzyme turnover at saturating substrate concentration has not been studied with V-ATPase, but has been a matter of a debate in studies of F_1 catalytic mechanism. Studies

Abbreviations: V-ATPase, proton-translocating vacuolar ATPase; 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-nitrobenzo-2-oxa-1,3-diazole; PEP, phosphoenolpyruvate; FCCP, carbonilcyanide p-triflouromethoxyphenylhydrazone.

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of the transition to multi-site catalysis during ATP synthesis by chloroplasts [17] and ATP hydrolysis by MF_1 [18], as well as studies on competition between TNP-ATP and ATP for binding to MF_1 [19] strongly support the bi-site model of multi-site catalysis according to which substrate binding to F_1 with a formation of the catalytic intermediate with two catalytic sites occupied results in a rapid enzyme turnover. Additional evidence supporting bi-site model has been recently provided by the results obtained when the catalytic site occupancy during multi-site ATP hydrolysis by MF_1 [20] and EcF_1 [21] has been measured using centrifugal filtration method and by the results obtained when inhibition of EcF_1 [22] and TF_1 [23] by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) has been studied in the presence of MgADP and/or MgATP. On the other hand, a support of the view that F_1 operates according to a tri-site mechanism, when rapid enzyme turnover can occur only after formation of an intermediate with all three catalytic sites occupied, currently comes mainly from the results obtained in two types of experiments. In one type, a tryptophan was inserted at the catalytic sites of F_1 as a reporter group and the catalytic-site occupancy was estimated using nucleotide-induced fluorescence quenching [24–26]. Another type of experiments involved single-molecule measurements of Cy3-ATP interaction with the $\alpha_3\beta_3\gamma$ subcomplex of TF_1 with the simultaneous observation of γ subunit rotation [27,28]. The results of both types of experiments were interpreted as supporting a tri-site model but the validity of such an interpretation has been recently challenged [21–23,29].

In the present study we investigated whether V-ATPase operates according to a bi-site or a tri-site mechanism by examining of how the enzyme was inhibited by NBD-Cl during ATP hydrolysis in a wide range of substrate concentration. NBD-Cl inhibits F_1- [30] and V-type [31–35] ATPases. Nucleotides protect F_1 [30] and V-ATPase [14,33,36–38] from inactivation by NBD-Cl, and nucleotide-protected labeling by NBD-Cl has been used to localize hydrolytic sites of V-ATPase to A subunits [14,36–39], while nucleotide concentration dependence of ECF_1 [22] and TF_1 [23] protection has been recently shown to support a bi-site catalytic mechanism. Cys-261 located in the glycine-rich loop (P-loop) of yeast A subunit has been suggested as the residue which modification by NBD-Cl leads to inhibition of V-ATPase [40].

The results obtained in the present study show that half-maximal protection of V-ATPase from inhibition by NBD-Cl occurs at MgATP concentrations that are significantly higher than the K_m values for the nucleotide regardless of whether or not ATP hydrolysis was coupled to energization of the vacuolar membrane. This pattern of protection is inconsistent with a tri-site model of catalysis and supports a bi-site mechanism for ATP hydrolysis coupled to a transmembrane transport of H^+ by V-ATPase.

2. Materials and methods

2.1. Materials

ATP, NADH, FCCP, triethanolamine, pyruvate kinase, and lyophilized lactate dehydrogenase were from Sigma. Mops and potassium phosphoenolpyruvate (PEP) were from Fluka, NBD-Cl was from Pierce, concanamycin A was from ALEXIS Biochemicals, and dimethyl sulfoxide was from Baker. Stock solutions of NBD-Cl (50 mM) were prepared in dry dimethyl sulfoxide and stored at −20 °C. pH of the stock solutions of ATP and PEP was adjusted to 7.0 with triethanolamine.

Vacuoles were prepared from the wild-type *Saccharomyces cerevisiae* yeast strain SF838–5Aα (*MATα, leu2–3112, ura-52, ade5*) according to [34] with a modification described by Liu and Kane [41]. At 37 °C, 0.1 μM concanamycin A inhibited ATPase activity of the vacuole preparations by 81–88% when assayed with 1 mM MgATP in the presence of 6 μM FCCP as described below. Under these conditions, concanamycin A-sensitive specific ATPase activity of the vacuoles was 1–2.5 U/mg (1 U is 1 μmol/min).

2.2. Inhibition of V-ATPase by NBD-Cl during ATP hydrolysis

To investigate the effect of MgATP on inhibition of V-ATPase by NBD-Cl, vacuolar vesicles (40–80 μg of protein per ml) were incubated for 15 s at room temperature (18–20 °C) in the medium containing 50 mM Mops/triethanolamine, pH 7.0, 2.2 mM Mg$(CH_3COO)_2$, 0.2 mM EDTA, 10 mM CH_3COOK, 1 mM PEP, 0.1 mg/ml pyruvate kinase, and MgATP as required in the absence or presence of 8 μM FCCP. Then NBD-Cl was added from 50-mM stock solution to obtain the desired final concentration (25–200 μM) and, after incubation for 0.5–10 min, ATPase activity was measured as described below using 40-μl aliquots of the reaction mixture.

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

$$A_t = A_1 + A_2 \times e^{-k't}, \quad (1)$$

where A_t is the vacuolar ATPase activity after incubation with NBD-Cl for a time t. The apparent second-order rate constants k of inhibition were then obtained by dividing the k'values by the concentration of NBD-Cl used.

2.3. ATPase activity assay

ATPase activity of vacuoles was measured spectrophotometrically [42] at 340 nm at 37 °C. The assay medium contained in a final volume of 1 ml 50 mM Mops/triethanolamine, pH 7.0, 0.2 mM EDTA, 3.2 mM Mg$(CH_3COO)_2$, 10 mM CH_3COOK, 1 mM ATP, 1 mM PEP, 0.3 mM NADH, 6 μM FCCP, 0.1 mg/ml pyruvate kinase, and 0.1 mg/ml lactate dehydrogenase. 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 vacuoles.

MgATP-concentration dependence of V-ATPase activity in vacuoles was measured at room temperature in the absence and presence of 6 μM FCCP, and at 37 °C in the presence of 6 μM FCCP in the medium containing 50 mM Mops/triethanolamine, pH 7.0, 0.2 mM EDTA, 2.2 mM Mg$(CH_3COO)_2$, 10 mM CH_3COOK, 1 mM PEP, 0.3 mM NADH, 0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, and 5 μM–2 mM MgATP. V-ATPase activity was calculated as a difference between activities measured in the absence and presence of 0.1 μM concanamycin A.

3. Results and discussion

Fig. 1A shows time-course of vacuolar ATPase activity inhibition by 25 μM (diamonds), 50 μM (circles), and 75 μM (hexagons) NBD-Cl. It is seen that within 10 min, the extent of inhibition reaches about 80%. Similar degree of inhibition by NBD-Cl was reported previously for yeast membrane-bound [34] and isolated V-ATPase [14,40]. Under our experimental conditions, incubation of vacuoles at room temperature in the absence of NBD-Cl resulted in slow decrease of ATPase activity that reached about 15% after 10-min incubation and about 25% after 30-min incubation. For this reason, to minimize NBD-Cl-independent decrease in activity, all the incubations with NBD-Cl were performed for no longer than 10 min. As it was shown with isolated V-ATPase [14], the pseudo first-order rate constants of inhibition of vacuolar ATPase activity obtained from the data of Fig. 1A are proportional to the NBD-Cl concentrations used (Fig. 1B). This result means that the rate of inhibition is limited by the rate of NBD-Cl binding to V-ATPase. The second-order

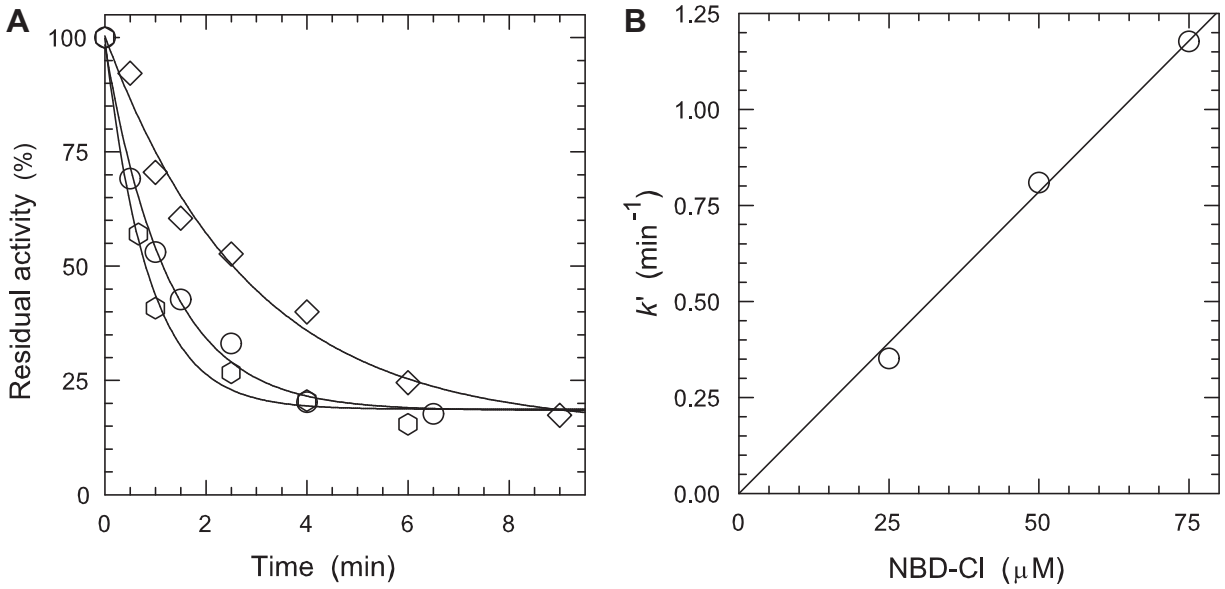

Fig. 1. Inhibition of vacuolar ATPase activity by NBD-Cl. (A) Time-course of inhibition in the presence of 0.025 mM (diamonds), 0.05 mM (circles), and 0.075 mM (hexagons) NBD-Cl. Vacuoles were incubated with NBD-Cl in the presence of FCCP and the ATPase activity was assayed as described in the Section 2. (B) Effect of NBD-Cl concentration on the pseudo first-order rate constants of vacuolar ATPase inhibition. The slop of the linear regression line is equal to $(1.65 \pm 0.25) \times 10^4\ M^{-1}\ min^{-1}$.

rate constant of NBD-Cl binding to V-ATPase obtained from the data of Fig. 1B is equal to $(1.65 \pm 0.25) \times 10^4\ M^{-1}\ min^{-1}$.

It was reported that, in the absence of Mg^{2+}, ATP protected V-ATPase in the vacuolar membranes from inhibition by NBD-Cl [14]. Half-maximal protection was observed in the presence of about 0.2 mM ATP. As the data of Fig. 2A show, MgATP also slows down inhibition of V-ATPase by NBD-Cl (in our experiments MgATP exhaustion was prevented due to presence of pyruvate kinase and PEP). Fig. 2B shows MgATP-concentration dependence of the apparent second-order rate constant k of the V-ATPase inhibition by NBD-Cl in the presence of uncoupler. Fitting the data of Fig. 2B to a hyperbolic equation

$$k = k_0/(1 + S/K_{1/2}), \quad (2)$$

where k_0 is the inhibition rate constant in the absence of MgATP, $K_{1/2}$ is the concentration of MgATP required for half-maximal decrease of k, and S is the concentration of MgATP, resulted in the best fit values of k_0 and $K_{1/2}$ of $(1.48 \pm 0.04) \times 10^4\ M^{-1}\ min^{-1}$ and 0.29 ± 0.04 mM, respectively. However, the line drawn using these best fit values (dashed line in Fig. 2B) significantly deviates from the experimental points. This indicates that complete protection of V-ATPase from inactivation by NBD-Cl requires MgATP binding at more than one catalytic site. A better fit (solid line in Fig. 2B) has been obtained using a model that assumes that MgATP binding

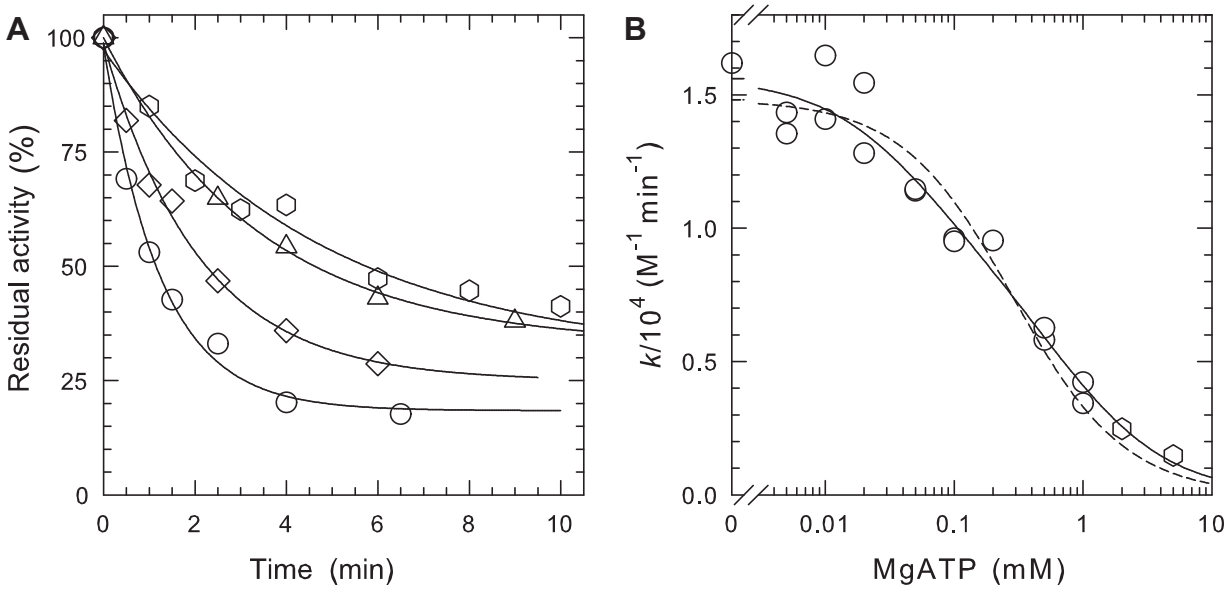

Fig. 2. Effect of MgATP on inhibition of V-ATPase by NBD-Cl in the presence of FCCP. (A) Time-course of vacuolar ATPase inhibition in the presence of FCCP by 0.05 mM (circles, diamonds, and hexagons) and 0.2 mM (triangles) NBD-Cl in the absence (circles) and presence of 0.1 mM (diamonds), 1 mM (hexagons), and 5 mM (triangles) MgATP. (B) Effect of MgATP concentration on the apparent second-order rate constant k of V-ATPase inhibition by NBD-Cl in the presence of FCCP. Values of k were obtained as described in the Section 2 using 0.05 mM (circles) and 0.2 mM (hexagons) NBD-Cl. Dashed line represents the best fit of the data to Eq. (2) ($k_0 = (1.48 \pm 0.04) \times 10^4\ M^{-1}\ min^{-1}$, and $K_{½} = 0.29 \pm 0.04$ mM). Solid line represents the best fit of the data to Eq. (3) (k_1, k_2, $K_{½,1}$, and $K_{½,2}$ are equal to $(0.7 \pm 0.5) \times 10^4\ M^{-1}\ min^{-1}$, $(0.86 \pm 0.5) \times 10^4\ M^{-1}\ min^{-1}$, 60 ± 50 μM, and 0.8 ± 0.6 mM, respectively).

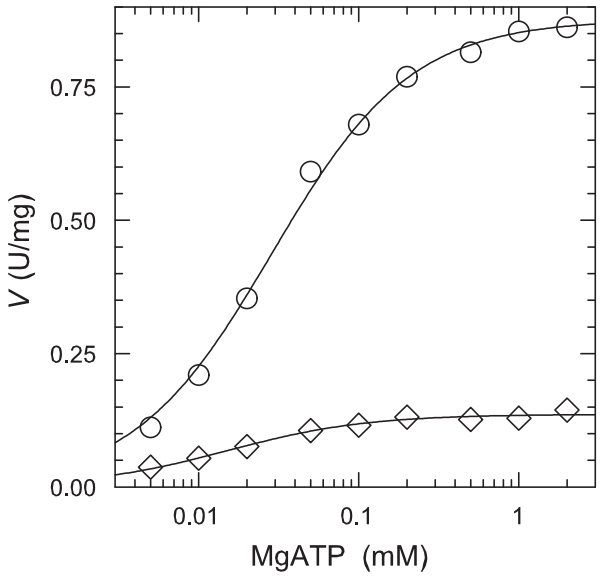

Fig. 3. MgATP-concentration dependence of V-ATPase activity in the absence (diamonds) and presence (circles) of FCCP. Concanamycin A-sensitive activity was assayed at room temperature as described in the Section 2. Lines represent the best fit of the data to the Michaelis–Menten equation (V_{max} = 0.88 ± 0.01 U/mg and K_m = 0.029 ± 0.002 mM in the presence of FCCP, and 0.136 ± 0.003 U/mg and 0.015 ± 0.002 mM, respectively, in the absence of FCCP).

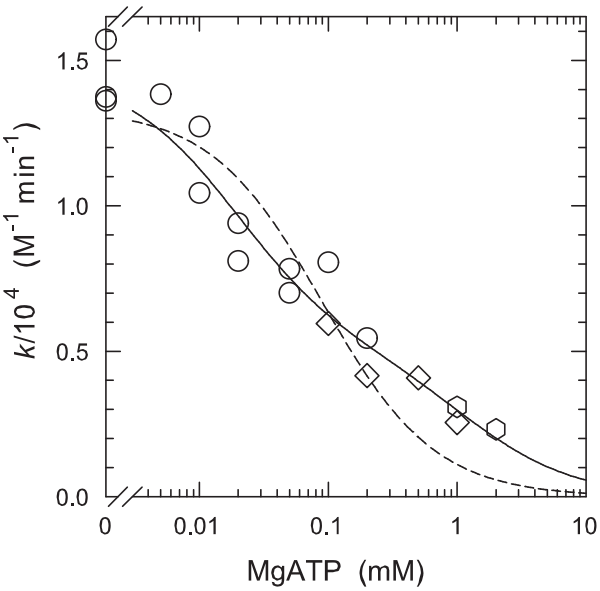

Fig. 4. Effect of MgATP concentration on the apparent second-order rate constant k of V- ATPase inhibition by NBD-Cl in the absence of FCCP. Values of k were obtained as described in the Section 2 using 0.05 mM (circles), 0.1 mM (diamonds) and 0.2 mM (hexagons) NBD-Cl. Dashed line represents the best fit of the data to Eq. (2) (k_0 = (1.34 ± 0.07) × 10^4 M^1 min^1, and $K_{½}$ = 0.09 ± 0.02 mM). Solid line represents the best fit of the data to Eq. (3) (k_1, k_2, $K_{½,1}$, and $K_{½,2}$ are equal to (0.9 ± 0.1) × 10^4 M^{-1} min^{-1}, (0.5 ± 0.1) × M^{-1} min^{-1}, 0.019 ± 0.008 mM, and 1.2 ± 0.9 mM, respectively).

to two catalytic sites is necessary for complete V-ATPase protection and is described by an equation

$$k = k_1/(1 + S/K_{1/2,1}) + k_2/(1 + S/K_{1/2,2}), \quad (3)$$

where ($k_1 + k_2$) is the inhibition rate constant in the absence of MgATP, $K_{1/2,1}$ and $K_{1/2,2}$ are the concentrations of MgATP required for half-maximal saturation of the catalytic sites where MgATP binding decreases the inhibition rate constant by k_1 and k_2, respectively. For the data shown in Fig. 2B, the best fit values of k_1, k_2, $K_{1/2,1}$, and $K_{1/2,2}$ are equal to (0.7 ± 0.5) × 10^4 M^{-1} min^{-1}, (0.86 ± 0.5) × 10^4 M^{-1} min^{-1}, 60 ± 50 μM, and 0.8 ± 0.6 mM, respectively.

Circles in Fig. 3 show MgATP-concentration dependence of concanamycin A-sensitive vacuolar ATPase activity measured at room temperature in the presence of uncoupler (that is under conditions used to obtain the data shown in Fig. 2B). Fitting the data to the Michaelis–Menten equation yielded the best fit values for V_{max} and K_m of 0.88 ± 0.01 U/mg and 29 ± 2 μM, respectively (for comparison, when ATPase assay was performed at 37 °C with the same vacuolar preparation, the V_{max} and K_m values obtained were equal to 2.63 ± 0.04 U/mg and 36 ± 2 μM, respectively (results not shown)).

Under the conditions used to obtain the results shown in Fig. 2B and Fig. 3 (circles), V-ATPase hydrolyzed ATP without formation of the proton-motive force across the vacuolar membrane due to presence of FCCP. As expected, when FCCP was omitted, lack of uncoupling resulted in a significant decrease of V-ATPase activity (Fig. 3, diamonds). In the absence of FCCP, the V_{max} and K_m values were equal to 0.136 ± 0.003 U/mg and 15 ± 2 μM, respectively. Fig. 4 shows the effect of MgATP on V-ATPase inhibition by NBD-Cl in the absence of FCCP. As with the data obtained in the presence of FCCP (Fig. 2B), the model with two binding sites (Eq. (3)) produced a better fit (solid line in Fig. 4, the best fit values of k_1, k_2, $K_{1/2,1}$, and $K_{1/2,2}$ equal to (0.9 ± 0.1) × 10^4 M^{-1} min^{-1}, (0.5 ± 0.1) × M^{-1} min^{-1}, 19 ± 8 μM, and 1.2 ± 0.9 mM, respectively) than the model with one binding site (Eq. (2), dashed line).

Both in the absence and presence of FCCP, the K_m values for MgATP are close to the respective values of $K_{1/2,1}$ but are significantly lower than the respective values of $K_{1/2}$ or $K_{1/2,2}$. These results mean that substrate saturation of a catalytic site responsible for rapid V-ATPase turnover is not sufficient to provide a complete protection of the enzyme from inhibition by NBD-Cl, and that the complete protection can be achieved only after substrate binding to an additional catalytic site. MgATP binding to this additional catalytic site results in no change in V-ATPase activity regardless of whether proton-motive force is present or not. This circumstance is inconsistent with a tri-site model of ATP hydrolysis that requires all three catalytic sites to be occupied at saturating substrate concentrations. On the other hand, the fact that V-ATPase remains sensitive to NBD-Cl in the presence of MgATP at concentrations sufficient to saturate the ATPase activity supports a bi-site mechanism that requires substrate binding to only two catalytic sites for a rapid enzyme turnover.

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