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Ligand-Dependent Structural Changes in the V_1 ATPase From *Manduca sexta*

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The response of V_1 ATPase of the tobacco hornworm *Manduca sexta* to Mg^{2+} and nucleotide binding in the presence of the enhancer methanol has been studied by $CuCl_2$-induced disulfide formation, fluorescence spectroscopy, and small-angle X-ray scattering. When the V_1 complex was supplemented with $CuCl_2$ nucleotide-dependence of A-B-E and A-B-E-D cross-linking products was observed in absence of nucleotides and presence of MgADP+Pi but not when MgAMP·PNP or MgADP were added. A zero-length cross-linking product of subunits D and E was formed, supporting their close proximity in the V_1 complex. The catalytic subunit A was reacted with *N*-4[4-[7-(dimethylamino)-4-methyl]coumarin-3-yl]maleimide (CM) and spectral shifts and changes in fluorescence intensity were detected upon addition of MgAMP·PNP, -ATP, -ADP+Pi, or -ADP. Differences in the fluorescence emission of these nucleotide-binding states were monitored using the intrinsic tryptophan fluorescence. The structural composition of the V_1 ATPase from *M. sexta* and conformational alterations in this enzyme due to Mg^{2+} and nucleotide binding are discussed on the basis of these and previous observations.

KEY WORDS: Vacuolar ATPase; V_1 ATPase; A_1 ATPase; F_1 ATPase; small-angle X-ray scattering; *Manduca sexta*.

INTRODUCTION

The vacuolar ATPases (V-type ATPases) are a family of ATP-dependent ion pumps found in two principal locations: the endomembranes and the plasma membranes. This family of ATPases is responsible for acidification of intracellular compartments and, in certain cases, ion transport across the plasma membrane of eucaryotic cells (Bowman and Bowman, 1997; Futai *et al.*, 2000; Kane, 2000; Nishi and Forgac, 2002). The V-ATPases are composed of a water-soluble V_1 ATPase and an integral membrane subcomplex, V_O. The integral V_O domain contains five different subunits in a stoichiometry of a_1:d_1:c_{4-5}:c'_1:c''_1 (Nishi and Forgac, 2002). ATP is hydrolyzed on the V_1 headpiece consisting of the nucleotide-binding subunits A and B (A_3:B_3), and the energy released during this process is transmitted to the membrane-bound V_O domain, to drive ion translocation. This energy-coupling occurs via the so-called "stalk" structure, an assembly of the V_1 and V_O subunits C−H and *a*, respectively, that forms the functional and structural interface (Müller and Grüber, 2003). A side view of the V_1 ATPase reveals that its structure is asymmetric with a headpiece and a single, compact stalk (Svergun *et al.*, 1998). Previously, we have obtained a three-dimensional structure of the V_1 ATPase without subunit C from the tobacco hornworm *Manduca sexta* at 1.8 nm resolution (Radermacher *et al.*, 2001). This showed that the A_3 and B_3 subunits alternate in an hexagonal arrangement as predicted before (Svergun *et al.*, 1998). At the upper end of the hexagonal barrel extensions can be observed, assumed to belong to the N-termini of subunit A. The hexagonal barrel formed by the subunits A and B encloses a cavity of approximately 3.2 nm in which part of the central stalk is

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asymmetrically located. The stalk region protrudes by approximately 6 nm from the surface of the A_3B_3-headpiece (Radermacher *et al.*, 2001).

The prevailing view is that ATP-hydrolysis in the V_1 headpiece is coupled to ion flow in V_O through movements of central stalk subunits (Aviezer-Hagai *et al.*, 2003; Hirata *et al.*, 2003; Müller and Grüber, 2003; Nishi and Forgac, 2002). Recent studies of the *M. sexta* V_1 ATPase have indicated that the positions of the stalk subunits E, F, G, and H are different whether Ca^{2+} + ATP or Ca^{2+} + ADP are bound to the catalytic sites (Grüber *et al.*, 2000a). Ca^{2+}-dependent enzyme activity of the *M. sexta* V_1 ATPase is strongly reminiscent of the effects of Ca^{2+} on the related F_1-ATPase (Hicks and Krulwich, 1986; McCarty and Racker, 1968; Ortiz Flores *et al.*, 1982; Schuster, 1979). Methanol prevents the inhibition of the Ca^{2+}-dependent ATPase activity of this V_1 by its product ADP (Gräf *et al.*, 1996). Like the chloroplast F_1-ATPase (McCarty and Racker, 1968), the *Manduca* V_1 prefers Mg^{2+} in the presence of methanol, thereby loosing the inhibition of ADP and increasing the hydrolytic activity to about 50–60%. Methanol appears to induce conformational changes in the enzyme, leading to enzymatic properties of V_1 that are similar to those of the membrane bound V_1V_O complex (Gräf *et al.*, 1996). We have investigated the structural alterations in the V_1 ATPase from *M. sexta* caused by Mg^{2+}- and nucleotide-binding by a variety of biophysical and biochemical methods. Altered $CuCl_2$-induced cross-link formation are discussed in the light of a structural model of the *M. sexta* V_1 complex.

EXPERIMENTAL PROCEDURES

Biochemicals

Chemicals for gel electrophoresis were obtained from Serva (Heidelberg, Germany). *N*-[4-[7-(dimethylamino)-4-methyl]coumarin-3-yl)]maleimide (CM) was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were at least of analytical grade and purchased from BIOMOL (Hamburg, Germany), Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany), Carl-Roth (Karlsruhe, Germany), or Serva (Heidelberg, Germany).

Purification of V_1 ATPase

Tobacco hornworms were reared as described at the web-site http://manduca.entomology.wisc.edu/manual/cover.html. The Manduca eggs were a generous gift of Dr. J. Schachtner, Philipps-University in Marburg and Prof. Trenczek, University of Giessen, Germany. The V_1 ATPase from *M. sexta* was isolated according to a modified protocol of Gräf *et al.* (1996) and Rizzo *et al.* (2003). The purity of the protein sample was analyzed by SDS-PAGE (Laemmli, 1970). SDS-gels were stained with Coomassie Brilliant Blue G250 or with silver (Damerval *et al.*, 1987). Protein concentrations were determined by the bicinchonic acid (BCA) assay (Pierce, Rockford, IL, USA). ATPase activity was measured as described previously (Rizzo *et al.*, 2003).

$CuCl_2$-Induced Cross-Link Formation

The protein was dialyzed overnight against a degassed buffer containing 20 mM Tris/HCl, pH 8.1 and 150 mM NaCl using a 10-kDa Spectra/Por dialysis membrane (Spectrum Laboratories, Canada) to remove loosely bound nucleotides and 2-mercaptoethanol. Such preparations retain 1.0–1.5 mol of tightly bound nucleotides as determined by FPLC-chromatography (Coskun *et al.*, 2002). After preincubation of the enzyme with 2 mM nucleotide for 5 min, cross-linking was induced by supplementation with 2 mM $CuCl_2$ in a buffer containing 20 mM Tris–HCl (pH 8.1), 150 mM NaCl, and 25% methanol on a rotary shaker (150 rpm) at 4°C for 30 min. The cross-linking reaction was stopped by addition of 10 mM EDTA, subsequently dissolved in DTT-free dissociation buffer and applied to an SDS-polyacrylamide gel as described above. The subunits involved in cross-linking were identified by SDS-PAGE. The new bands, including the cross-link products, were cut out and divided into small pieces. After addition of dissociation buffer with 2% ß-mercaptoethanol and 50 mM dithiothreitol (DTT), gel pieces were heated at 95°C for 10 min and centrifuged for 5 min at 13, 500 rpm using a mini-spin centrifuge (Eppendorf). The supernatant was applied instantly to an SDS-polyacrylamide gel as described above.

Labeling V_1 ATPase by CM and Fluorescence Measurements

Before labeling with *N*-[4-[7-(dimethylamino)-4-methyl]coumarin-3-yl)]maleimide (CM), V_1 was depleted of nucleotides as described above. The enzyme was labeled with 20 μM CM for 10 min in 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl (buffer A). Excess label was removed by dialysis against buffer A using a 300-kDa Spectra/Por dialysis membrane (Spectrum Laboratories, Canada). Fluorescence emission spectra of CM-bound V_1

and the intrinsic tryptophan fluorescence in the presence and absence of 2 mM of different nucleotides and 25% methanol were recorded at 10°C using a *Varian Cary Eclipse* spectrofluorimeter. CM-bound V_1 samples were excited at 365 nm, and the emission was recorded from 400 to 580 nm with excitation and emission bandpasses set to 5 nm. The excitation wavelength for the intrinsic fluorescence measurements was 280 nm, and the emission was detected over the range of 300–380 nm. Excitation and emission bandpass were set to 5 nm.

X-Ray Scattering Experiments and Data Analysis

The synchrotron radiation X-ray scattering data were collected following standard procedures on the X33 camera (Boulin *et al.*, 1986; Koch and Bordas, 1983) of the EMBL on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY) using multiwire proportional chambers with delay line readout (Gabriel and Dauvergne, 1982). Solutions with protein concentration of 4.5 and 8 mg/ml were measured. At a sample-detector distance of 3.9 m and a wavelength $\lambda = 0.15$ nm the ranges of momentum transfer $0.13 < s < 2.2\ \text{nm}^{-1}$ was covered ($s = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle). The data were normalized to the intensity of the incident beam and corrected for the detector response; the scattering of the buffer was subtracted and the difference curves were scaled for concentration using the program SAPOKO (Svergun and Koch, unpublished). The maximum dimension, D_{max}, of V_1 with and without nucleotides, the distance distribution function $p(r)$ and the radii of gyration, R_g, were computed with the indirect Fourier transform program *GNOM* (Svergun, 1992).

RESULTS

Nucleotide-Dependent Cross-Linking in the V_1 ATPase

The V_1 ATPase from *M. sexta* can adopt at least two conformations, depending on whether CaATP or CaADP is present (Grüber *et al.*, 2000a). In order to describe possible alterations in the V_1 ATPase more fully, we have now examined the enzyme by $CuCl_2$-induced cross-linking after addition of MgAMP·PNP, -ATP, -ADP+Pi, or -ADP in the presence of the enhancer methanol. When the V_1, free of loosely bound nucleotides, was supplemented with $CuCl_2$ four defined new bands (I–IV) and several A-B oligomers at higher molecular mass were observed in SDS polyacrylamide electrophoresis gels (Fig. 1(A)). To analyze the bands of the new cross-link formations the products were cut out from the gel, and prepared for a second SDS-PAGE in the presence of β-mercaptoethanol and DTT (see "Experimental Procedures"). As shown in Fig. 1(B), the products I–IV contain the subunits D-E (I), A-B-E (II), A-B-D-E (III), and A_2-B-E (IV). It is of particular interest that subunit D does not contain Cys-residues. Inspection of the amino acid sequence of this

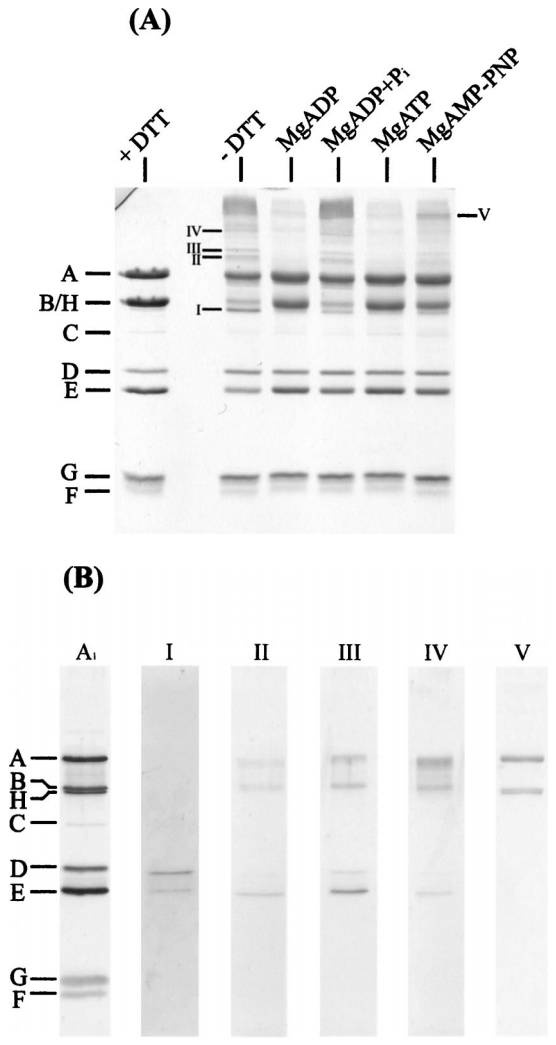

Fig. 1. Cross-linking of V_1 ATPase depending on Mg^{2+} and nucleotide-binding. (*A*) The enzyme was supplemented with 2 mM MgAMP·PNP, -MgATP, -MgADP, -MgADP+Pi in the presence of 25% of methanol for an incubation time of 5 min at 4°C, followed by addition and incubation of 2 mM $CuCl_2$ for 30 min at 4°C. The reaction was stopped by addition of 10 mM EDTA. V_1 without nucleotides is shown in *lanes* 1 and 2. Eight micrograms of enzyme were applied per lane after addition of DTT-free sample buffer or after addition of 1 mM DTT (*lane* 1). Cross-linking products are marked I–V. The gel was stained with Coomassie Blue G250. (B) Two-dimensional SDS-PAGE analyzis of the cross-linking products I–V. The new bands I–V in the gel from panel A were cut out, treated as described under "Experimental Procedures" and applied onto a 17.5% total acrylamide and 0.4% cross-linked acrylamide gel. The gel was stained with silver.

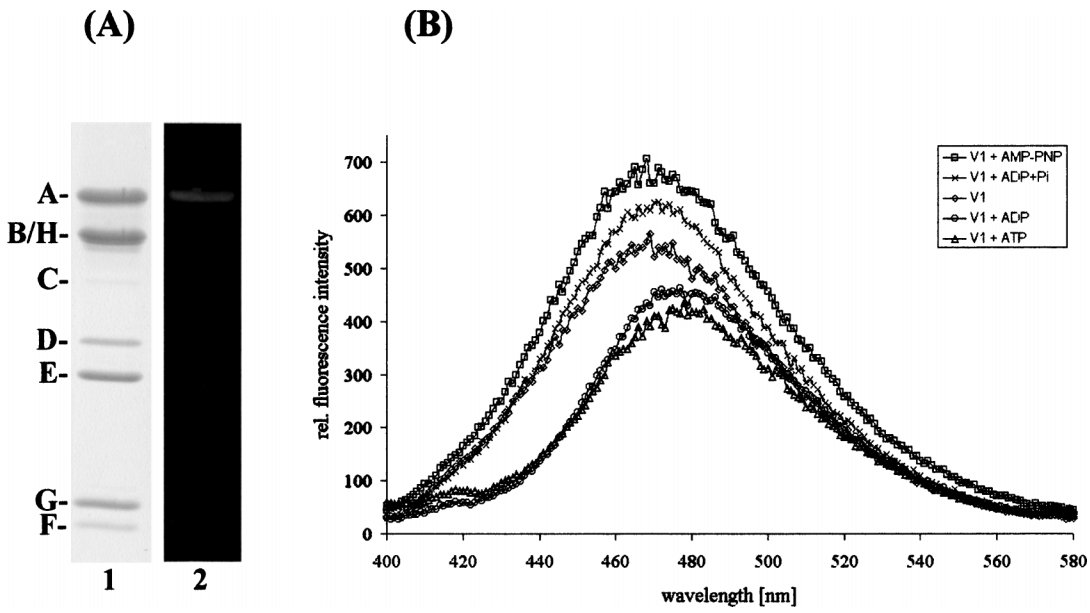

Fig. 2. Labeling of subunit A and nucleotide-induced fluorescence changes of the CM bound V_1 from *M. sexta*. The enzyme was reacted with CM as described under "Experimental Procedures" and applied to a 17.5% total acrylamide and 0.4% cross-linked SDS polyacrylamide gel. (*A*) The gel stained with Coomassie Blue G250 (*left*) and the fluorogram of the same gel (right). (*B*) The fluorescence emission spectrum of the CM-labeled V_1 ATPase was measured with a protein concentration of 400 nM and a 1:1 ratio of Mg^{2+} to nucleotide at 10°C in the presence of 25% of methanol as described in "Experimental Procedures." The enzyme was diluted in 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl and preincubated with 2 mM of MgAMP·PNP (*curve* □), MgATP (*curve* Δ), MgADP (*curve* ○), and MgADP+Pi (*curve x*) on ice. *Curve* ◇, V_1 ATPase in the absence of nucleotides. The spectra were recorded at λ_{ex} of 365 nm over a range of 400–580 nm with the emission and excitation slits at 5 nm.

subunit reveals the presence of two His- ($His_{30,174}$) and Tyr-residues ($Tyr_{112,119}$) (Merzendorfer *et al.*, 2000), respectively, both candidates to form a thioether-bridge with a cysteinyl residue. Such covalent link of the sulfur of a Cys- with an imidazole nucleus of a His-residue or with a Tyr-residue has been identified as an essential formation in the tyrosinase from *Neurospora crassa* (Lerch, 1982) and the galactose oxidase from *Dactylium dendroides* (Ito *et al.*, 1991). When the enzyme was incubated with 2 mM MgADP (in a 1:1 ratio) at 4°C before $CuCl_2$-treatment no significant disulfide-formation was formed. In the presence of MgADP + Pi the bands I–IV were visible, with a lower amount of band I (D-E product), when compared to the cross-link formation of the nucleotide-depleted enzyme. When V_1 was suspended in MgATP only a small amount of cross-link product I (subunits D and E) was obtained. Addition of the noncleavable nucleotide analogue AMP·PNP + Mg^{2+} resulted in a D-E formation and the new band V, including the subunits A_2-B-H.

Spectroscopic Investigations of the V_1 ATPase

To further characterize the structural changes due to ligand-binding described above, the catalytic subunit A was specifically labeled using the fluorescent label CM, as visualized on the polyacrylamide gel in Fig. 2(A). The activity of the enzyme dropped from 1.9 to 1.3 μmol of ATP hydrolyzed per milligram of protein per minute by the incorporation of CM. Figure 2(B) shows the fluorescence spectrum of the CM labeled V_1 ATPase, which was freed of loosely bound nucleotides (*curve* ◇) as described under "Experimental Procedures." For comparison, addition of 2 mM MgATP to the protein causes the signal to decrease and to shift to longer wavelength (*curve* Δ). Addition of MgADP (*curve* ○) displayed a spectrum similar to that obtained with MgATP but with a slightly higher fluorescence maximum. In contrast, V_1 in the presence of MgADP+Pi (*curve x*) gave a higher fluorescence intensity compared to the nucleotide depleted enzyme. Addition of the noncleavable nucleotide analogue AMP·PNP+Mg^{2+} (*curve* □) caused a further increase of the fluorescence signal, indicating that the MgATP bound enzyme hydrolyzed most of the ATP to ADP during the measurements, which is in agreement with the results of Cu^{2+}-induced cross-link formations described above. Taken together, the data illustrate that the fluorescence spectrum of CM bound to subunit A is sensitive to nucleotide binding.

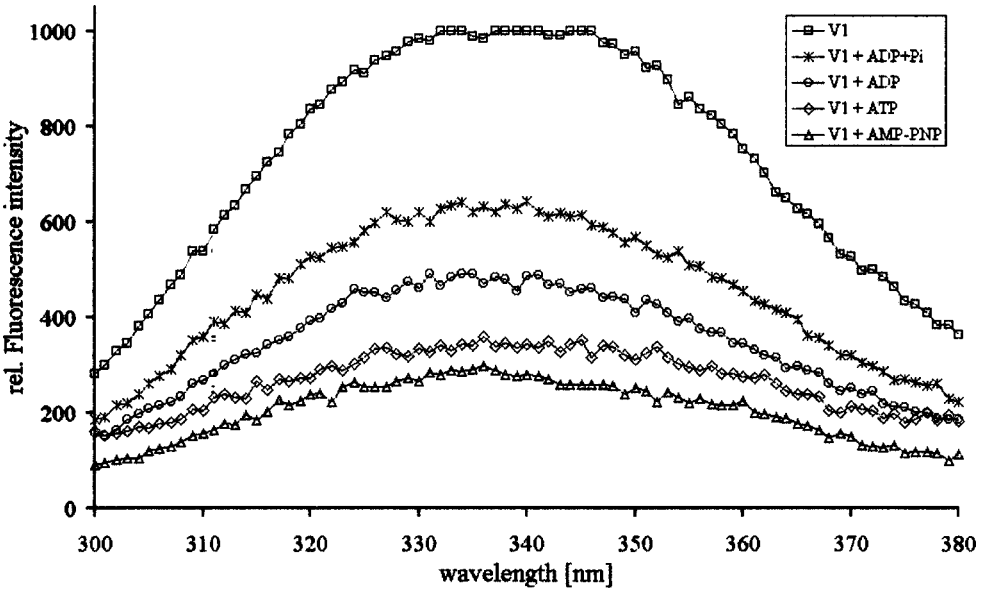

Fig. 3. Fluorescence emission spectra of nucleotide-depleted and nucleotide-bound V_1. The emission spectra were recorded 5 min after addition of 2 mM MgAMP·PNP (*curve* Δ), MgATP (*curve* $\diamond$), MgADP (*curve* $\circ$), or MgADP+Pi (*curve x*) in the presence of 25% methanol at $\lambda_{em} = 280$ nm with emission and excitation slits at 5 nm. Curve $\boxplus$ represents the spectra of the nucleotide-depleted enzyme.

To obtain additional information on possible alterations of secondary structures in the V_1 complex after nucleotide-binding the fluorescence emission of the enzyme with and without ligands (curve $\boxplus$) was monitored using the intrinsic tryptophan fluorescence (Fig. 3). The V_1 from *M. sexta* contains 21 tryptophans: 6, 1, 8, and 6 in the subunits nucleotide-binding subunits A (Gräf *et al.*, 1992), B (Novak *et al.*, 1992), and the stalk subunits C and H (Merzendorfer *et al.*, 2000), respectively. Addition of MgADP+Pi (*curve x*) resulted in a quenching (up to 40%) of the intrinsic fluorescence. The fluorescence intensity drops further by the addition of the ligands MgADP (60%; *curve* $\circ$) and MgATP (70%; *curve* $\diamond$). The addition of MgAMP·PNP (*curve* Δ) decreased the quantum yield markedly (up to 80%) and shifted the spectrum to slightly shorter wavelengths.

Effect of Substrate-Binding Studied by X-Ray Solution Scattering

The conformational changes were further investigated by small angle X-ray scattering (SAXS) in order to determine whether they affect the overall structure of the V_1 complex. The experimental solution scattering curve of the nucleotide-depleted V_1 ATPase and the corresponding distance distribution function are presented in Fig. 4(A) and (B). The maximum dimension of the nucleotide-free V_1-ATPase is 22.0 ± 0.3 nm and its radius of gyration, as deduced from Guinier plots and from the distance distribution function $p(r)$, is 6.2 ± 0.2 nm. When 2 mM MgADP, -ADP+Pi, -ATP, or MgAMP·PNP was added to the protein solution, the radii of gyration of the complexes were determined to be to 6.2 ± 0.2 nm, 6.1 ± 0.2 nm, 6.1 ± 0.3 nm, and 6.1 ± 0.3 nm, respectively (see Table I). The scattering curves (not shown) nearly coincide. The maximum dimension of the unligated or ligated V_1 ATPase was unchanged (22.0 ± 0.3 nm), indicating that the quaternary structure of the enzyme is not altered.

DISCUSSION

Methanol is known to be an effective enhancer of Mg^{2+}-dependent ATPase-activity of F_1-ATPases from bacteria, chloroplasts and mitochondria (Hicks and Krulwich, 1986; McCarty and Racker, 1968; Flores *et al.*, 1982; Schuster, 1979). The native cytosolic V_1 ATPase of *M. sexta*, which is the object of the present study, displays different patterns of activity. In the absence of methanol, the enzyme is dependent on Ca^{2+}, and is inhibited by the hydrolytic product, ADP. By comparison, in the presence of methanol, the V_1 prefers Mg^{2+} over Ca^{2+}, is not longer inhibited by ADP and its hydrolytic activity increases to about 50–60%. Methanol thus appears to induce a conformational change in V_1, resulting in enzymatic properties which are similar to those of the V_1V_O ATPase (Gräf *et al.*, 1996). Our results provide

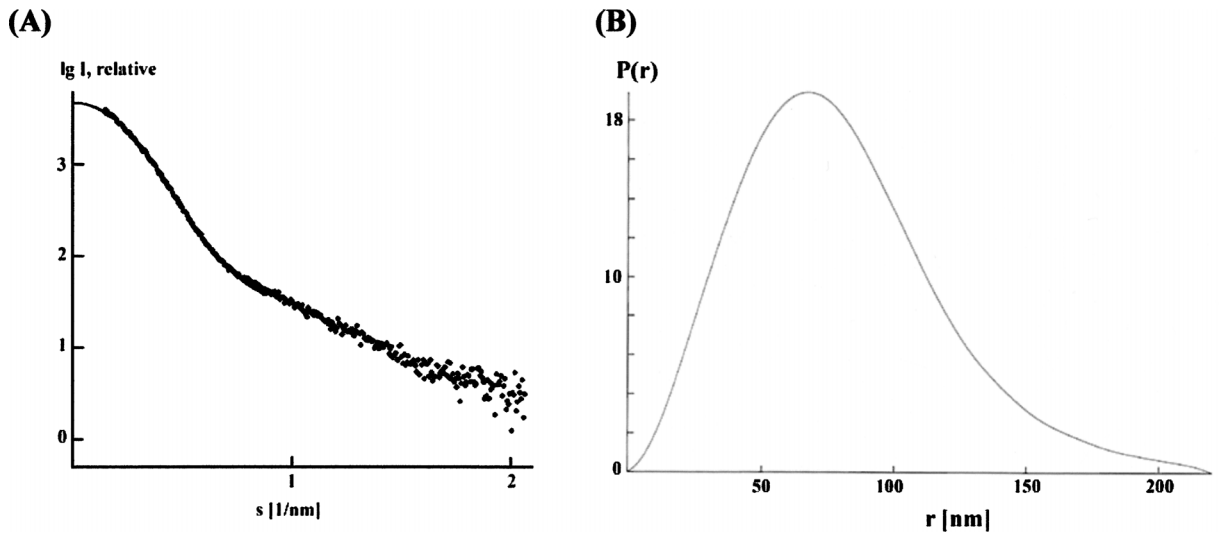

Fig. 4. (A) Experimental scattering curve and (B) distance distribution function of nucleotide-depleted V_1 ATPase from *M. sexta*.

new data on the structure of the V_1 ATPase from *M. sexta* and help to describe nucleotide-dependent conformational changes occurring in the Mg^{2+}-dependent complex. Using Cu^{2+} to induce zero-length cross-links, nucleotide-induced rearrangement of the major subunits A and B and the stalk subunits D, E, and H were obtained. $CuCl_2$-treatment of the nucleotide-depleted enzyme results in A-B, A-B-E, A-B-D-E, A_2-B-E, and D-E formation. The formation of A-B oligomers reflects the proximity of these subunits, which are shown to alternate around a cavity in a hexameric fashion (Radermacher *et al.*, 2001; Svergun *et al.*, 1998), thereby locating the nucleotide-binding sites at their interfaces (Nishi and Forgac, 2002). The close proximity of the stalk subunit E to the major subunits A and B demonstrated by the A-B-E, A_2-B-E products is consistent with the isolation of an A-B-E-subcomplex after treatment of the *M. sexta* V_1 ATPase with the detergent lauryldimethylamine oxide (LDAO) (Rizzo *et al.*, 2003). The nucleotide-dependent cross-linked products A-B-D-E and D-E are an important finding. A homologous cross-linked formation consisting of the subunits D and E of the V-ATPase from clathrin-coated vesicles has also been generated using the cross-linking reagent 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Xu *et al.*, 1999). Furthermore, the neighborhood of both subunits inside the V-ATPase has also been shown by the use of the bifunctional reagent 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP) (Adachi *et al.*, 1990). In the data presented, a significant amount of D-E complex is formed in the absence of nucleotides and after addition of MgADP+Pi whereas a lower amount is formed in the presence of MgATP or -AMP·PNP. Conformational alterations in E and rearrangements of this subunit in the V_1 complex due to binding of CaATP and -ADP were described recently (Grüber *et al.*, 2000a). In these experiments we have shown that in the presence of CaATP the cross-linked products A-E-F and B-H were formed, whereby additon of CaADP resulted in E-F and E-G complexes (Grüber, 2000). The data imply that the V_1 ATPase of *M. sexta* displays different subunit arrangements during Ca^{2+}-dependent ATPase activity and methanol-induced Mg^{2+}-dependent hydrolytic activity. Thus subunit D of the *M. sexta* V_1 ATPase is unnecessary for either Ca^{2+}-dependent ATPase activity (Gräf *et al.*, 1996; Rizzo *et al.*, 2003) and for the methanol-induced Mg^{2+}-dependent hydrolytic activity (Gräf *et al.*, 1996; Grüber *et al.*, 2000a), the formation of a nucleotide-dependent D-E complex implies that subunit E alters its arrangement relative to subunit D during enzyme activity. In this context it should be mentioned, that rotational movements of subunit D of the A_3B_3DF subcomplex of the *Thermus thermophilus*

Table I. Radii of Gyration, R_g, of the V_1 ATPase From *M. sexta* Dependent on Nucleotide Conditions

Added nucleotides	R_g (nm)
None	6.2 ± 0.2
2 mM MgATP	6.1 ± 0.3
2 mM MgADP	6.2 ± 0.2
2 mM MgADP+Pi	6.1 ± 0.2
2 mM MgAMP·PNP	6.1 ± 0.3

H^+-ATPase has been demonstrated (Imamura *et al.*, 2003). However, this ATPase, which is responsible for ATP synthesis, has been classified into A-type ATPases found in the cytoplasmic membranes of most archaea and some bacteria (Hilario and Gogarten, 1998; Ihara *et al.*, 1992).

Our results also support the proposed structural model of the *M. sexta* V_1 in which subunit E is a central ATPase-coupling element inside the enzyme, thereby in close proximity to subunit D (Rizzo *et al.*, 2003). The D-E zero-length cross-link formation emphasizes the presence of a single compact stalk in the cytosolic V_1, as shown in the three-dimensional models of the *M. sexta* V_1 ATPase (Radermacher *et al.*, 2001; Svergun *et al.*, 1998). Such structural composition of an A_3:B_3 hexamer and one stalk domain of the soluble V_1 is consistent with a hexameric arrangement of the major nucleotide-binding subunits and a single compact stalk domain in the closely related A_1 and F_1 ATPases as shown by X-ray solution scattering (Grüber, 2000; Grüber *et al.*, 2000b; Svergun *et al.*, 1998) and X-ray crystallography (Gibbons *et al.*, 2000; reviewed in Pedersen *et al.*, 2000).

Incorporation of the fluorescent label CM as a reporter group into the catalytic binding site A of V_1 as described above, demonstrates changes of the secondary structure in this major subunit. Addition of the nonhydrolyzable ATP analog MgAMP·PNP causes a significant fluorescent enhancement, which is lower in the presence of MgADP+Pi. In contrast, the presence of MgADP results in a quenching of the signal and red shift, suggesting that structural changes in and around the bound CM occur in response to ATP binding and subsequent bond cleavage to ADP and Pi. Strong evidence for secondary structural alterations in the V_1 due to Mg^{2+} and nucleotide-binding are provided by the marked decrease of intrinsic fluorescence. However, the X-ray solution scattering results indicate that the radii of gyration (R_g) of the nucleotide depleted and Mg^{2+} plus nucleotide-bound enzyme coincide within experimental error. This is in agreement with the recently determined R_g-values 6.2 $\pm$ 0.06 nm, 6.12 $\pm$ 0.04 nm of the *M. sexta* V_1 complex before and after addition of CaATP (Grüber *et al.*, 2000a). These findings imply that the overall envelope of the V_1 complex does not change significantly after substrate-binding and hence also not during hydrolytic activity.

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