Cross-talk in the A1-ATPase from Methanosarcina mazei Gö1 Due to Nucleotide Binding*

Received for publication, October 30, 2001, and in revised form, February 18, 2002

Published, JBC Papers in Press, February 19, 2002, DOI 10.1074/jbc.M110407200

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Changes in the A2B2CDF-complex of the Methanosarcina mazei Gö1 A1-ATPase in response to ligand binding have been studied by small-angle x-ray scattering, protease digestion, fluorescence spectroscopy, matrix-assisted laser desorption ionization time-of-flight mass spectrometry, and CuCl2-induced disulfide formation. The value of the radius of gyration, Rg, increases slightly when MgATP, MgADP, or MgADP + Pγi (but not MgAMP-PNP) is present. The nucleotide-binding subunits A and B were reacted with N-4-[7-(dimethylamino)-4-methylcoumarin-3-yl]maleimide, and spectral shifts and changes in fluorescence intensity were detected upon addition of MgAMP-PNP, MgATP, MgADP + Pγi, or MgADP. Trypsin treatment of A1 resulted in cleavage of the stalk subunits C and F, which was rapid in the presence of MgAMP-PNP but slow when MgATP or MgADP were added to the enzyme. When A1 was supplemented with CuCl2 a clear nucleotide dependence of an A-D cross-linking product was generated in the presence of MgADP and MgATP but not when MgAMP-PNP or MgADP + Pγi was added. The site of cross-link formation was located in the region of the N and C termini of subunit D. The data suggest that the stalk subunits C, D, and F in A1 undergo conformational changes during ATP hydrolysis.

The membrane-integrated archaeal A1A2-ATPase (A1A2BCDEFGHIKx), the exact stoichiometry of the subunits is unknown, like the bacterial F1F0-ATPase (αβ3γδεabcx) and the eucaryotic V1V0-ATPase (A1B2CDFEGH_ac_de), possesses an intrinsic domain (A1), containing the catalytic sites, and an intrinsic domain (A2), involved in ion translocation (1–3). The primary structure of the archaeal ATPase is very similar to that of the V-ATPase, but its function, as an ATP-synthase, is more similar to that of F-ATPases (2, 4–6). Electron microscopy has shown that the major nucleotide-binding subunits A and B of the A1/N1 and the corresponding β and α subunits of the F1 form an alternating hexagonal arrangement (7–9) around a central mass (10, 11). The hexameric headpiece is attached to the A1F0V0 part by only one stalk (3). Recent three-dimensional structures of F1 (12) and V1 (13) confirm these features and show the stalk part extending from and therefore partly composed of F1 (γει; mitochondrial subunit nomenclature (12)) and V1 subunits (C–H).

The A2B2CDF complex of the Methanosarcina mazei Gö1 A1-ATPase, which is investigated here, consists of a ~96 Å long headpiece and an 84 Å high and 60 Å diameter stalk as shown by small-angle x-ray scattering (14). A comparison of the central stalk of this A1 complex with the F1- and V1-ATPase indicates different shapes and lengths of these domains (3, 14) which account for linking catalytic events in the headpiece with ion pumping through the membrane portion. In particular, the F1-ATPase has a significantly shorter stalk than A1, ~40–45 Å long and 50–55 Å wide (12, 15). The prevailing view is, however, that ATP hydrolysis in the A1 headpiece is coupled to ion flow in A1 through movements of the central stalk subunit(s) (2) as visualized by optical microscopy for F1 by fixing this enzyme on a surface and attaching either an actin filament or a bead to the γ subunit to mark its orientation (reviewed in Ref. 16). Although the energy-transducing mechanism of A-ATPase is thought to be similar to that of the F-ATPase, evidence for structural alterations during coupling in the A-ATPase has been lacking. Here we describe this phenomenon in the A1-ATPase by a variety of biophysical and biochemical methods. Altered overall dimensions of the A1 complex, fluorescence changes, trypsin susceptibility, and CuCl2-induced cross-link formation between various subunits of A1 are discussed in the light of different ligand-dependent states.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were at least of analytical grade and were obtained from Bionol (Hamburg, Germany), Merck, Promega (Madison, WI), Sigma, or Serva (Heidelberg, Germany).

Purification of A1-ATPase—The A1-ATPase from Methanosarcina mazei Gö1 was obtained from Escherichia coli strain DRK expressing the A1-ATPase genes A–G on a multicopy vector pTL2 (17). The enzyme was isolated by gel permeation chromatography followed by ion exchange chromatography, as will be described elsewhere.1 For solution x-ray scattering experiments (see below) the enzyme was subsequently applied onto a Sephacryl S-300 HR column (10/30, Amersham Biosciences) equilibrated in 50 mM Tris-HCl (pH 6.9), 150 mM NaCl and subjected to gel permeation chromatography (FPLC)2 in order to isolate a homogeneous and nucleotide-depleted A1 complex (14). The purity and homogeneity of

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1 T. Lemker and V. Müller, manuscript in preparation.

2 The abbreviations used are: FPLC, fast protein liquid chromatography; CM, N-4-[7-(dimethylamino)-4-methyl]coumarin-3-yl]maleimide; AMP-PNP, adenosine 5′-(β,γ-iminodithiotriphosphate; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DTT, dithiothreitol; SAXS, by small-angle x-ray scattering.
the protein sample was analyzed by Native-PAGE (18) and SDS-PAGE (19). SDS gels were stained with Coomassie Brilliant Blue G-250. Protein concentrations were determined according to Lowry et al. (20). ATPase activity was measured as described previously (14, 21).

CuCl2-induced Cross-link Formation—Bound nucleotides were removed by passing the A1 complex through a size exclusion column (Superdex 200 HR 10/30, Amersham Biosciences) equilibrated in 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl. After preincubation of the enzyme with 5 mM nucleotide for 5 min, cross-linking was induced by supplementation with 2 mM CuCl2 in a buffer containing 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl on a rotary shaker (450 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 Western blotting (22) using antisera against subunit A and B as described previously (17) and mass spectrometric analysis. For the latter the cross-linked bands were cut out from the SDS-polyacrylamide gel and destained with a solution of 25 mM ammonium bicarbonate and 50% acetonitrile for 12 h. The gel band was cut into pieces of 1 mm2, which were washed three times with acetonitrile, dried for 30 min in a speed-vacuum concentrator, and digested according to a procedure modified from Hellmann et al. (23) and Roos et al. (24). For MALDI mass spectrometry, aliquots of 0.5 μl of the digested solution were applied to a target disc and allowed to dry in the air. Subsequently, 0.5 μl of matrix solution (1% w/v α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid) was applied to the dried sample and also allowed to dry. Spectra were obtained using a Bruker Biflex III MALDI-TOF mass spectrometer. The protein fragments were identified using programs from the University of California, San Francisco (rafael.ucsf.edu/cgi-bin/msfit), the ProFound program of Rockefeller University (prowl.rockefeller.edu/cgi-bin/ProFound), the PepSearch program of the EMBL in Heidelberg (www.mann.emb- heidelberg.de/Services/PeptideSearch/FR_peptideSearchForm.html), and TagIdent available on the ExPASy WWW server.

**Labeling A1-ATPase by CM2 and Fluorescence Measurements—** Before labeling with N-[(7-dimethylamino)-4-methyl]coumarin-3-yl]maleimide (CM), A1 was depleted of nucleotides as described above. The enzyme was labeled with 50 μM CM for 10 min in 20 mM Tris-HCl (pH 6.9) and 150 mM NaCl (buffer A). Excess label was removed by one pass through a Sephadex G-25 spin column, equilibrated in buffer A. Fluorescence emission spectra of CM-bound A1 in the presence and absence of 5 mM of different nucleotides were recorded at 10 °C using an SLM-Aminco 8100 spectrophotometer. Protein samples were excited at 365 nm, and the emission was recorded from 410 to 560 nm with excitation and emission bandpasses set to 4 nm. In order to determine the amount of hydrolyzed MgATP after the fluorescent measurement, a 200-μl portion of the sample was denatured with 20 μl of 14% perchloric acid and cooled on ice for 15 min. The denatured protein was then pelleted by centrifugation at 5000 rpm for 2 min (25, 26). The supernatant was transferred to a microcentrifuge tube containing 3 μl of 5 M K2CO3 for neutralization. The nucleotide content of 100 μl of samples from this supernatant was determined by FPLC (Econo-System, Bio-Rad) using a molecular mass standard. The eluents were monitored by absorption at 254 nm, and the amounts were determined by integration of absorption peaks, calibrated with ATP and ADP standards.

**Trypsin Digestion Studies—** A1-ATPase was incubated at a concentration of 8 μg with trypsin in a ratio of 900:1 (w/w) in 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl in the absence or presence of 5 mM nucleotide at 30 °C. Trypsin cleavage was stopped by addition of the protease inhibitor Pefabloc SC (8 mM). Peptides were separated by SDS-PAGE (14).

**X-ray Scattering Experiments and Data Analysis—** The synchrotron radiation X-ray scattering data were collected following standard procedures on the X33 camera (27–29) of the EMBL on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY) using multiwire proportional chambers with delay line readout (30). Solutions with protein concentrations of 4.3 and 7.9 mg/ml were measured. At sample detector distances of 3.9 and 1.4 m and a wavelength λ = 0.15 nm, the ranges of momentum transfer 0.14 < s < 2.1 nm−1 were covered (s = 4π sin θ/λ, 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. The maximum dimension, Dmax, of the A1-ATPase samples, their distance distribution function p(s) and radii of gyration, Rg, were computed by the indirect Fourier transform program GNOM (31, 32).

The molecular masses of the solutes were estimated by comparison with the forward scattering of a reference solution of bovine serum albumin.

![Fig. 1. Native-PAGE and experimental scattering curves of the A2B2CDF-complex of the A1-ATPase. A, the homogeneity and purity of the A1-complex has been proven by Native-PAGE (lane 1) before exposure to x-rays using a gradient of 4–15%. Lane 2, the V31-ATPase and the V31(-C) complex from M. sexta with apparent molecular masses of 600 and 560 kDa (13), respectively, were used as molecular mass standard. B, x-ray small-angle scattering curves of the A1 complex before (curve 1) and after the addition of 5 mM MgAMP-PNP (curve 5), MgATP (curve 4), MgADP + P, (curve 3), and MgADP (curve 2), collected at 10 °C. The curves are displaced by 1 logarithmic unit for better visualization.](Image)

### Table I

| Added nucleotides | Rg (nm) |
|-------------------|--------|
| None              | 5.02 ± 0.1 |
| 5 mM MgATP        | 5.23 ± 0.1 |
| 5 mM MgADP        | 5.14 ± 0.1 |
| 5 mM MgADP + P    | 5.21 ± 0.1 |
| 5 mM MgAMP PNP    | 4.92 ± 0.1 |

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The molecular masses of the solutes were estimated by comparison with the forward scattering of a reference solution of bovine serum albumin.

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3 D. I. Svergun and M. H. J. Koch, unpublished data.
RESULTS

Effect of Substrate Binding Studied by X-ray Solution Scattering—Previously, we have characterized the A1-ATPase from M. mazei Go1 by small-angle x-ray scattering (SAXS) and determined the maximum dimension (18.0 ± 0.1 nm) and the radius of gyration (Rg, 5.03 ± 0.1 nm) (14). Here SAXS was used to investigate possible changes of the quaternary structure of A1 due to substrate binding. Fig. 1 displays a native gel and the scattering profile of A1, free of loosely bound nucleotides and an ATPase activity of ~8.0 μmol of ATP hydrolysis per mg of enzyme per min. The radius of gyration (5.02 ± 0.1 nm) is in agreement with previous results (14). However, when 5 mM MgADP, MgADP + P, or MgATP was added to the protein solution, the radius of gyration of the complexes increased to 5.14 ± 0.1, 5.21 ± 0.1, and 5.23 ± 0.1 nm, respectively (see Table I). In the presence of the unhydrolyzable ATP analogue, AMP-PNP, the enzyme had a slightly lower Rg = 4.92 ± 0.1 nm. The maximum dimension of the unligated or ligated A1-ATPase remained the same (18.0 ± 0.1 nm).

CM Labeling of A1 and Spectroscopic Investigations—To characterize further the structural changes described above, the nucleotide-binding subunits A and B were specifically labeled using the fluorescent label CM, as visualized on the polyacrylamide gel in Fig. 2A. The higher intensity of CM bound at subunit A may be due to the fact that subunit A includes five Cys residues (Cys28, Cys65, Cys173, Cys255, and Cys372 (33)) and thereby more possible sulfhydryl groups reacting with the maleimide. The activity of the CM-bound enzyme was not altered by the chemical modification. Fig. 3 shows the fluorescence spectrum of the CM labeled A1-ATPase, which was freed of bound nucleotides (Fig. 3, curve a) as described under “Experimental Procedures.” For comparison, addition of 5 mM MgATP to the protein (in a 1:1 ratio) causes the signal to increase and to shift to shorter wavelength (Fig. 3, curve △). Addition of MgADP (Fig. 3, curve □) gave a spectrum similar to that obtained with MgATP but with a lower fluorescence maximum. In contrast, A1-ATPase in the presence of MgADP + P_i (Fig. 3, curve ○) displayed a lower fluorescence intensity and a small blue shift. Interestingly, addition of the non-cleavable nucleotide analogue AMP-PNP (Fig. 3, curve ◊) caused an increase of the fluorescence signal, indicating that the MgATP-bound enzyme is catalyzing ATP hydrolysis during the measurements. This is also confirmed by the fact that 85% of the added MgATP is still present after the fluorescence measurement, as determined by FPLC. The results presented indicate that the fluorescence spectrum of CM bound to subunits A and B is sensitive to nucleotide binding.

FIG. 2. Labeling of subunits A and B by CM. 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 G-250; B, the fluorogram of the same gel.

FIG. 3. Nucleotide-induced fluorescence changes of the CM-bound A1-ATPase. The fluorescence emission spectra of the A1-ATPase was measured with a protein concentration of 200 nM and a 1:1 ratio of Mg^2+ to nucleotide at 10 °C. The enzyme was diluted in 20 mM Tris-HCl (pH 6.9) and 150 mM NaCl and preincubated with 5 mM MgAMP-PNP (curve ◊), MgATP (curve △), MgADP (curve □), and MgADP + P_i (curve ○) on ice. Curve ▲, A1-ATPase in the absence of nucleotides. The spectra were recorded at λ_em of 365.1 nm over a range of 410–560 nm with the emission and excitation slits at 4 nm.
Cross-linking of A₁ Subunits Induced by CuCl₂—Intersubunit cross-linking is a useful method for establishing the relative position of subunits (34–36). Disulfide bond formation was mediated by Cu²⁺. Fig. 4A illustrates the results of cross-linking of the A₁-ATPase with CuCl₂ under different nucleotide conditions. When the enzyme was incubated with 5 mM MgAMP-PNP at 4 °C before Cu²⁺ treatment, two new bands (I and V) involving subunit(s) B (I) and A-B (V) were generated, as indicated by Western blotting with antibodies to subunits A and B (Fig. 4B). MALDI mass spectrometry confirmed that band I derived from subunit B by identifying the peptides Ala₁₀₂–Arg₁₂₀, Gly₂₇₁–Arg₂₈₆, and Ala₃₆₆–Arg₃₇₉. When A₁ was suspended in MgADP/H₁₁₀₀₁, the cross-link product I and a low amount of band V are obtained. In the presence of MgATP the bands I and V increased, and the new bands III, IV, and VI–VIII appeared, including the subunits A-A (III) and A-A-D (IV), respectively, and several forms of A-B oligomers (V–VIII). The A-A-D (IV) formation, which cannot be cleaved by DTT, was analyzed more precisely by MALDI mass spectrometry (Fig. 5).

Ten peptides were unequivocally identified as bands deriving from either the N or C terminus of subunit D, together with five peptides of subunit A (see Table II). However, we were not able to monitor unequivocally the specific peptide of each subunit forming the linkage. This might be due to the incompleteness of in-gel tryptic digestion of the polypeptides (37), which covers only 47.8 and 23.9% of the D and A sequence, respectively. The presence of MgADP leads to a slight decrease of the A-A-D (IV) formation and additionally to several closely spaced bands running just above band VIII, including A-B and A-A formations as shown by antibody blotting (Fig. 4B). Consistently, the staining intensity of the A and B bands decreased in parallel with the occurrence of these A-B and A-A oligomer bands. These results rule out that MgATP was completely converted into MgADP or MgADP/H₁₁₀₀₁ before cross-link formation under the conditions used, and it can be concluded that the differences in the fluorescence spectra seen above depend on whether MgATP or MgADP is bound in the nucleotide-binding site of the A₁-ATPase. As shown by MALDI mass spectrometry, addition of MgADP leads to an intrinsic cross-link of the contaminant DnaK (band II). A cross-linked B product (I) and an A-B oligomer (see Fig. 4A and B) were observed.

**Fig. 4.** Characterization of cross-linking products in A₁-ATPase due to nucleotide binding. A, the enzyme was supplemented with 5 mM MgAMP-PNP, MgATP, MgADP, and MgADP + P₁, or with the A₁-ATPase inhibitor dienestrol (0.4 μM) for an incubation time of 5 min at 4°C, followed by addition and incubation of 2 mM CuCl₂ for 30 min at 4°C. The reaction was stopped by addition of 10 mM EDTA. The control containing no nucleotides is shown in lanes 6 and 8. 8 μg of enzyme were applied per lane after addition of DTT-free dissociation buffer or after addition of 1 mM DTT (lane 8). Cross-link products are marked by I–VIII. The gel was stained with Coomassie Blue G-250. B, immunoblot of the same samples with polyclonal rabbit antisera directed against the A and B subunits of the A₁ complex. C, two-dimensional SDS-PAGE to analyze the cross-link products I and V. Lane 6 of the gel in A were cut out, destained, soaked in buffer consisting of 20 mM Tris-HCl (pH 6.9) and 150 mM NaCl, 50 mM DTT, and 0.5% SDS, and placed onto a 17.5% total acrylamide and 0.4% cross-linked acrylamide gel.
gomer formation (V) were also formed in the presence of the A₁ inhibitor dienestrol (17) and under atmospheric oxygen without CuCl₂ as shown by two-dimensional SDS-PAGE (Fig. 4C). Addition of DTT after CuCl₂ treatment reversed cross-linking of the oxidized A₁ complex.

Nucleotide-dependent Trypsin Treatment of the A₁-ATPase—Recently, limited tryptic digestion of A₁ has shown that the subunits A–C are cleaved most rapidly, leading to the fragments A₁–4, B₁, C₁, followed by F, which becomes cleaved into fragment F₁, whereby subunit D remains shielded by the complex (14). The cleavage products and their intensities were the same as observed upon tryptic treatment of A₁ in the presence of 5 mM MgAMP-PNP (Fig. 6A), with subunit F cleaved into fragment F₁ after 20 min (data not shown). In contrast, there is slow cleavage of these subunits with MgATP or MgADP. Subunit C, which completely vanishes in the absence (14) or presence of MgAMP-PNP after 50 min, is slowly cleaved when MgATP or MgADP is added to the enzyme. There is, however, a slight difference in cleavage of this stalk subunit depending on whether MgATP or MgADP is bound. Quantitation of the scanned C-bands indicate that 43 and 59.4% of this subunit remained after 70 min when MgATP or MgADP, respectively, is bound to the enzyme (Fig. 6B). Proteolysis of A₁ after addition of MgADP + Pᵢ leads to a time-dependent cleavage pattern in which only 4.7% of subunit C remained after 70 min. Furthermore, under this nucleotide condition the subunits A, B, and F also become more accessible to trypsin than in the presence of MgATP or MgADP. Interestingly, the cleavage pattern of the MgADP + Pᵢ-bound A₁-complex is quite comparable with the feature seen when MgAMP-PNP is present. This is in agreement with the results of Cu²⁺-induced cross-link formations described above when MgADP + Fᵢ or MgAMP-PNP are bound to the enzyme.

**Discussion**

X-ray solution scattering was used to investigate the influence of nucleotide binding to the quaternary structure of the A₁-ATPase from *M. mazei* GsI. Binding of the uncleaved MgATP (obtained by adding MgAMP-PNP) causes a slight decrease in the radius of gyration of this complex, consistent with the observation on the closely related F₁,F₀-ATPase from *E. coli*, where the diameter of the F₁ complex decreases upon MgAMP-PNP binding as determined by three-dimensional reconstructions (38). In contrast, addition of the hydrolyzable MgATP, MgADP + Pᵢ, and MgADP increase the radii of gyration. Conformational changes of the quaternary and tertiary structure of the related F₁ due to nucleotide binding are in line with the most recent crystallographic model of bovine F₁-ATPase, indicating changes of the quaternary and tertiary structure of the complex (39). Depending on the nucleotide bound to one of the catalytic β subunits significant changes of the lower part of the nucleotide binding domain and the C-terminal domain have been observed. Superposition of the so-called β₂₂₂, β₁₁₁, and β₁₂₁–Pᵢ subunits of the bovine F₁-ATPase (39) indicate a 33° rotation of the C-terminal domain of β₁₂₁–Pᵢ when compared with β₁₁₁ and significant changes in backbone torsion angles in regions of the nucleotide binding domain. Incorporation of the fluorescent label CM into the nucleotide-binding site A and B of A₁, as described above, provides strong evidence for rearrangements of these subunits, which also suggests that the small but systematic differences observed in SAXS are significant. Addition of MgADP causes a significant fluorescent enhancement and blue shift, whereas the binding of MgAMP-PNP only increases the signal. In contrast, the presence of MgADP + Pᵢ results in a quenching of the signal, suggesting that structural changes in and around the bound CM occur in response to ATP binding and subsequent bond cleavage to ADP and Pᵢ.

The nucleotide-induced rearrangement of the major subunits A and B is also confirmed by the quantity of Cu²⁺-induced A-B dimers or the formation of A-B oligomers. The formation of A-B oligomers reflects the proximity of these subunits, which are proposed to alternate around a cavity in a hexameric fashion, thereby locating the nucleotide-binding sites at their interfaces (2, 40). Significantly, A-A dimers can be observed even though separated by an intervening B subunit. Close proximity of the related α and β subunits of the F₁-ATPase has also been demonstrated by α-α or β-β products formed via disulfide bridges of the N or C terminus, respectively (35, 41). Like in the closely related V-ATPases, the A₁ subunit A contains a region of 80–90 additional amino acids near the N terminus (5, 6, 33). This extension is assumed to be located at the top of the three A subunits, forming protuberances as described for the V-ATPase (10, 13, 42). Therefore, depending on nucleotide binding to the catalytic A subunits, these protuberances might come in close contact thereby facilitating an A-A formation.

A key finding of the present study is that subunit D can be cross-linked to the catalytic A subunit depending on nucleotide binding. The A-D formation occurs after addition of MgADP and to a small amount in the presence of the hydrolyzable MgATP but not in the presence of MgADP + Pᵢ, MgAMP-PNP, or the absence of nucleotides. The interaction between the subunits A and D involves the N and C termini of D, indicating their close proximity to the catalytic A subunit. It is of particular interest that neither of the termini of D contain Cys.

**Table II**

| Subunit | Start residue | End residue | Expected mass | Measured mass | Delta mass | Sequence |
|---------|---------------|-------------|---------------|---------------|-----------|----------|
| A       | 199           | 210         | 1338.7        | 1338.7        | 0.0       | LTPeKFLVtgqR |
|         | 341           | 357         | 1911.8        | 1911.8        | 0.0       | LEMPeGGeyGPYASLar |
|         | 341           | 357         | 1927.8        | 1927.8        | 0.0       | LEMPeGGeyGPYASLar^a |
|         | 433           | 451         | 1940.9        | 1940.8        | -0.1      | IMKAI/mKWGDAAMDALK^a |
|         | 376           | 401         | 2571.2        | 2571.3        | +0.1      | GSiTaIGAVSPGDeSePfVQntlR |
| D       | 11            | 19          | 1073.3        | 1072.5        | -0.8      | SelINLKKK |
|         | 19            | 28          | 1127.3        | 1126.5        | -0.2      | KIlKsesghK |
|         | 184           | 194         | 1517.8        | 1517.6        | -0.4      | YIrFmLeemer^a |
|         | 20            | 33          | 1629.0        | 1628.7        | -0.3      | IklKsesghKlKMK^a |
|         | 187           | 199         | 1748.9        | 1748.9        | 0.0       | FmLemEmertFr^a |
|         | 32            | 49          | 2210.6        | 2211.0        | +0.4      | MkrKdlLeffKIlneAr^a |
|         | 143           | 164         | 2564.0        | 2564.1        | +0.1      | ITaAaeLtMkKlLDeIekTk^a |
|         | 143           | 165         | 2720.2        | 2720.1        | -0.1      | ITaAaeLttMkKlLDeIekTk^a |
|         | 35            | 61          | 3095.6        | 3095.5        | -0.1      | DGLlEffKILNerAvntDlAdAFAk |
|         | 174           | 199         | 3337.9        | 3338.5        | +0.6      | VIFLlIDTmkYrIFmLemErertFr^a |

^a Oxidized Met.
residues and that the cross-linked formation cannot be disrupted by reducing agents, which rules out that the A-D product would be generated by disulfide bond formation. Inspection of the amino acid sequence of the peptides from subunit D reveals the presence of a His27 and a Tyr184 residue at the N and C termini of this subunit, respectively, both candidates to form a thioether bridge with a cysteinyl residue. Such covalent linkage of the sulfur of a Cys with an imidazole ring of a His residue or with a Tyr residue has been identified as an essential formation in the tyrosinase from N. crassa (43) and the galactose oxidase from Dactylium dendroides (44), respectively, and also occurred in cross-linking studies of the

FIG. 6. Electrophoretic analysis and quantitation of nucleotide-dependent trypsin cleavage of A1-ATPase. A, the A1-complex was incubated for 5 min with 5 mM MgAMP-PNP, MgATP, MgADP, or MgADP + Pi before trypsin cleavage (ratio of 900:1 (w/w)) for the indicated times. Proteolysis was stopped by addition of the protease inhibitor Pefabloc SC to a final concentration of 8 mM. Samples were electrophoresed on a 17.5% total acrylamide and 0.4% cross-linked acrylamide gel and stained with Coomassie Blue G-250. B, quantitation of the nucleotide-dependent cleavage of subunit C. Relative intensities of the protein bands of subunit C at each time point and under the nucleotide conditions described above were determined by scanning the gel of A with an HP (ScanJet 4c) flat-bed scanner. The intensity of each protein band was digitized and calculated by the program AIDA 2.40 (advanced image analyzer; RAYTEST). The symbols labeling the different nucleotide conditions used are shown in the inset.
Nucleotide-dependent Movements in the A1-ATPase

ATP synthase from E. coli (45). This close proximity of subunits A and D would allow coupling between the catalytic site events in A via D into the central stalk, which provides the physical and structural linkage between the AβBβ headpiece and the ion-conducting complex (2, 3). Secondary structure analysis of D predicted α-helical N and C termini (33, 45), as shown for both termini of subunit γ of F-ATPase (12, 47, 48), which has been proposed as a structural and functional homologue of subunit D (3, 14, 33, 46). The x-ray structure revealed that the α-helical N and C termini of γ intercalate into the cavity of the αβ2β2 assembly of F0 (12, 48, 49), thereby linking two differently occupied catalytic subunits, βTP (triphosphate-containing) and βDP (diphosphate-containing) (39).

Taken together, the results above provide several lines of evidence suggesting that, depending on the nucleotides bound to them, subunits A and B of the A1-ATPase change conformation and/or their interactions with structural alterations in the stalk subunits C, D, and F. The nucleotide dependence suggests also that there is a tight interaction of the D subunit at its catch region with the A subunit upon MgADP binding, which is broken when MgAMP-PNP is bound. Such binding followed by release may play a part in coupling the catalytic sites with the stalk region and the membrane-bound ion channel.

Acknowledgment—We thank R. Genswein, Universit"at Mainz, for excellent technical assistance.

REFERENCES

1. Perzov, N., Pudler-Karavani, V., Nelson, H., and Nelson, N. (2001) FEBS Lett. 504, 223–228
2. Sch"afer, G., Engelhard, M., and M"uller, V. (1999) Mol. Biol. Rev. 63, 570–620
3. Gruber, G., Wieczorek, H., Harvey, W. R., and M"uller, V. (2001) J. Exp. Biol. 204, 2597–2605
4. Bogart, J. P. (1995) Trends Ecol. Evol. 10, 147–151
5. Hilario, E., and Gogarten, J. P. (1998) J. Mol. Biol. 266, 703–715
6. Marin, L., Fares, M. A., Gonz"ales-Candelas, F., Barrio, E., and Moya, A. (2001) J. Mol. Biol. 30, 17–28
7. L"ubben, M., L"undorf, H., and Sch"afer, G. (1988) Biol. Chem. Hoppe-Seyler 369, 1259–1266
8. Rademacher, M., Ruiz, T., Harvey, W. R., Wieczorek, H., and Gruber, G. (1999) FEBS Lett. 453, 383–386
9. Gogol, E. P., Lucken, U., Bork, T., and Capaldi, R. A. (1989) Biochemistry 28, 4709–4716
10. Gruber, G., Rademacher, M., Ruiz, T., Godovac-Zimmermann, J., Canas, B., Kleine-Kohlebrecher, D., Huss, M., Harvey, W. R., and Wieczorek, H. (2000) Biochemistry 39, 8609–8616
11. Gogol, E. P., Aggeler, R., Sagermann, M., and Capaldi, R. A. (1989) Biochemistry 28, 4717–4724
12. Gibbons, C., Montgomery, M. G., Leslie, A. G. W., and Walker, J. E. (2000) Nat. Struct. Biol. 7, 1055–1061
13. Rademacher, M., Ruiz, T., Wieczorek, H., and Gruber, G. (2001) J. Struct. Biol. 135, 26–37
14. Gruber, G., Svergun, D. I., Coskun, U., Lemker, T., Koch, M. H. J., Sch"agger, H., and M"uller, V. (2000) Biochemistry 40, 1890–1896
15. Gruber, G. (2000) J. Bioenerg. Biomembr. 32, 341–346
16. Yoshida, M., Muneyuki, E., and Hisahori, T. (2001) Nat. Rev. Mol. Cell. Biol. 2, 669–677
17. Lemker, T., Ruppert, C., St"oger, H., Wimmers, S., and M"uller, V. (2001) Eur. J. Biochem. 268, 3744–3750
18. Sch"agger, H., and von Jagow, G. (1991) Anal. Biochem. 199, 223–231
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21. Heininen, J. K., and Laiti, R. J. (1981) Anal. Biochem. 113, 313–317
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
23. Poom, M., Kosnik, V., Poznanovic, S., and Godovac-Zimmermann, J. (1998) J. Biol. Chem. 273, 984–981
24. Bouligh, D. A., Brown, E. L., Saar, J. D., and Allison, W. S. (1988) J. Biol. Chem. 263, 14043–14060
25. Gruber, G., Hausrath, A., Sagermann, M., and Capaldi, R. A. (1997) FEBS Lett. 410, 165–168
26. Koch, M. H. J., and Bordas, J. (1983) Nucl. Instrum. Methods 208, 461–469
27. Boulin, C., Kempf, R., Koch, M. H. J., and McLaughlin, S. M. (1986) Nucl. Instrum. Methods 212, 320–323
28. Gabriel, A., and Dauvergne, P. (1982) Nucl. Instrum. Methods 212, 223–224
29. Svergun, D. I. (1993) J. Appl. Crystallogr. 26, 258–267
30. Sotstedt, C., Purnesek, F., Mack, J. R., and Feig, L. A. (1988) Acta Crystallogr. Sect. A 44, 244–250
31. Wilms, R., Freiberg, C., Wegerle, E., Meier, I., Mayer, F., and M"uller, V. (1996) J. Biol. Chem. 271, 18843–18852
32. Achauer, B., Haughton, M. A., and Capaldi, R. A. (1995) J. Biol. Chem. 270, 9185–9191
33. Tsuoda, S. P., Muneyuki, E., Amano, T., Yoshida, M., and Nishi, J. (1999) J. Biol. Chem. 274, 5701–5706
34. B"ottcher, B., Bertsche, I., Reuter, R., and Grauer, P. (2000) J. Mol. Biol. 296, 449–457
35. Menz, R. I., Walker, J. E., and Leslie, A. G. (2001) Cell 106, 331–341
36. Sch"afer, G., and Meyerings-Vos, M. (1992) Biochim. Biophys. Acta 1101, 232–235
37. Ogilvie, I., Aggeler, R., and Capaldi, R. A. (1997) J. Biol. Chem. 272, 16652–16656
38. Wilken, S., Vasilievna, E., and Forzac, M. (1999) J. Biol. Chem. 274, 31804–31810
39. Lorch, K. (1982) J. Biol. Chem. 257, 6414–6419
40. Ito, N., Phillips, S. E. V., Stevens, C., Ogil, Z. B., McPherson, M. J., Keen, J. N., Yadav, R. D. S., and Kowless, P. F. (1991) Nature 350, 87–90
41. Watts, M. D., Zhang, Y., Fillingame, R. H., and Capaldi, R. A. (1995) FEBS Lett. 365, 225–230
42. M"uller, V., Ruppert, C., and Lemker, T. (1999) J. Bioenerg. Biomembr. 31, 15–27
43. Rodgers, A. J. W., and Wilce, M. C. J. (2000) Nat. Struct. Biol. 7, 1051–1054
44. Hausrath, A. C., Gruber, G., Matthews, B. W., and Capaldi, R. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13697–13702
45. Hausrath, A. C., Capaldi, R. A., and Matthews, B. W. (2001) J. Biol. Chem. 276, 47227–47232