Evidence for Major Structural Changes in the *Manduca sexta* Midgut V₁ ATPase Due to Redox Modulation

A SMALL ANGLE X-RAY SCATTERING STUDY* 

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The shape and overall dimensions of the oxidized and reduced form of the V₁ ATPase from *Manduca sexta* were investigated by synchrotron radiation x-ray solution scattering. The radius of gyration of the oxidized and reduced complex differ noticeably, with dimensions of 6.20 ± 0.06 and 5.84 ± 0.06 nm, respectively, whereas the maximum dimensions remain constant at 22.0 ± 0.1 nm. Comparison of the low resolution shapes of both forms, determined ab initio, indicates that the main structural alteration occurs in the head piece, where the major subunits A and B are located, and at the bottom of the stalk. In conjunction with the solution scattering data, decreased susceptibility to tryptic digestion and tryptophan fluorescence of the reduced V₁ molecule provide the first strong evidence for major structural changes in the V₁ ATPase because of redox modulation.

Ion-translocating, vacuolar-type, ATPases (V-ATPases), which use ATP to acidify intracellular compartments and energize plasma membranes, consist of a hydrophilic, catalytic complex, V₀, and a hydrophobic, ion-translocating channel complex, V₁ (1, 2). In the larval midgut of the tobacco hornworm, *Manduca sexta*, the V₀ complex is made up of four subunits with apparent molecular masses of 100 (a), 40 (d), 20 (e) and 17 (c) kDa. The hydrophilic portion, V₁ ATPase, consists of eight distinct subunits of molecular mass 67 (A), 56 (B), 54 (H), 40 (C), 32 (D), 28 (E), 16 (G), and 14 kDa (F). Three interdigitation copies each of the nucleotide-binding subunits A and B form a head piece at the top of the molecule, whereas the remaining V₁ subunits form a stalk connecting the head piece to the V₀ complex (3).

Several mechanisms have been proposed for the regulation of V-ATPases, including disassembly and reassembly of peripheral V₁ and integral V₀ complexes (4–7), regulation of parallel ion channels that compensate for the electrogenicity of the V-ATPase (8, 9), changes in the degree of coupling between ATP hydrolysis and proton pumping (9, 10), and reversible disulfide bond interchange between conserved cysteine residues near the catalytic site (reviewed in Ref. 11). Studies on the bovine clathrin-coated V-ATPase have shown that disulfide bond formation between the conserved residues Cys²⁵⁴ and Cys³⁵³ of the catalytic A subunit leads to reversible inactivation of the enzyme (12, 13). Such a reversibly inactivated, disulfide-bonded state has been found in significant fractions of V-ATPase in native clathrin-coated vesicles (12). Disulfide bonds at the catalytic site of the V-ATPase are also induced by the nitric oxide-generating reagent S-nitrosoglutathione (14). This is consistent with the finding that in *Neurospora crassa* oxidizing and reducing agents have inhibitory and stabilizing effects, respectively, on the enzyme (15). Moreover, a mutation in the cysteine biosynthetic pathway in yeast leads to defective vacuolar acidification, which can be corrected by a C261V mutation, suggesting that disulfide bond formation can cause inhibition of V-ATPase activity in vivo (16).

In the present paper we show for the first time that reducing the V₁ ATPase of *M. sexta* leads to significant structural changes in the head and bottom pieces of the complex. Altered mobility of the V₁ subunits in polyacrylamide gels, decreased tryptophan fluorescence, and trypsin susceptibility are discussed in the light of these structural changes.

**EXPERIMENTAL PROCEDURES**

Materials—Chemicals for gel electrophoresis were obtained from Serva (Heidelberg, Germany). Trypsin used for in-gel digestion was obtained from Promega (Madison, WI). All other chemicals were at least of analytical grade and were obtained from Merck, Sigma, or Serva.

**Purification of the V₁ ATPase**—The V₁ ATPase from *M. sexta* midgut was isolated as described previously (7, 18). The V₁ complex was then applied onto a Superdex HR200 (10/30) column, equilibrated in 20 mM Tris/HCl (pH 8.1), and 150 mM NaCl (buffer A) with or without the addition of 1 mM CaATP, to remove sulfhydryl-reducing reagents that were introduced during the isolation of the enzyme. Protein concentrations were determined with Amido Black (19). SDS-polyacrylamide gel electrophoresis was performed with 17.5% total acrylamide (T) and 0.4% cross-linked acrylamide (C) or with a 10–16% linear gradient and 1% (C). The protein was supplemented with buffer (187.5 mM Tris/HCl (pH 8.1), and 150 mM NaCl (buffer A) with or without the addition of 1 mM CaATP), to remove sulfhydryl-reducing reagents that were introduced during the isolation of the enzyme. Protein concentrations were determined with Amido Black (19). SDS-polyacrylamide gel electrophoresis was performed with 17.5% total acrylamide (T) and 0.4% cross-linked acrylamide (C) or with a 10–16% linear gradient and 1% (C). The protein was supplemented with buffer (187.5 mM Tris/HCl (pH 8.1), and 150 mM NaCl (buffer A) with or without the addition of 1 mM CaATP). Protein bands on gels were stained with Coomassie Brilliant Blue R (20). ATPase activity was measured in the presence of 50 mM Tris-Mops* (pH 8.1), 20 mM KCl, 1 mM ATP, 1 mM MgATP, and 25% methanol (7).

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* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 431 and Habilitandenstipendium Grant GR 1475/6-1, National Institutes of Health Grant AI 22444, International Association for the Promotion of Cooperation with Scientists from the Independent States of the Former Soviet Union Grant 98-1115 (to D. I. S. and M. H. J. K.), and European Union Biotechnology Program Grant BIO4-CT97-2143 (to D. I. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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**The abbreviations used are**—Mops, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; DAM, dummy atoms model; MALDI, matrix-assisted laser desorption ionization; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

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Fluorescence Measurements—The intrinsic tryptophan fluorescence was recorded at room temperature using an SLM-AMINCO 8100 spectrophotometer. Protein samples were excited at 280 nm, and the emission was recorded over the range of 300–370 nm. Excitation and emission bandpasses were set at 4 nm.

X-ray Scattering Experiments and Data Analysis—The synchrotron radiation x-ray scattering data were collected following standard procedures on the X33 camera (21–23) of the EMBL on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY) using multiwire proportional chambers with delay line readout (24). Solutions with protein concentrations of 4.5 and 10 mg/ml were measured. At a sample-to-detector distance of 4.0 m and a wavelength $\lambda = 0.15$ nm, the range of the momentum transfer $0.14 < q < 2.2$ nm$^{-1}$ was covered ($s = 4\pi \sin\theta/\lambda$, where $2\theta$ 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 SAPIAOKO. The maximum dimensions $D_{\text{max}}$ of the nonreduced $V_1$ ATPase, which was isolated as described above in buffer A (including 20 mM Tris/HCl, pH 8.1) and 150 mM NaCl at 30°C, were determined automatically by DAMMIN from the outer part of the curve (28, 30).

The latter is performed starting from a random configuration that both forms of the $V_1$ ATPase are monomeric in solution. This indicates that the particle becomes more compact in the reduced state than in the oxidized state of the enzyme. As a preliminary to x-ray solution scattering experiments, the intrinsic tryptophan fluorescence of the oxidized and reduced state of the $V_1$ ATPase was monitored using the intrinsic tryptophan fluorescence, to determine whether or not the inhibition coincides with conformational changes in the enzyme. The $V_1$ ATPase from $M. sexta$ contains 21 tryptophans: 6, 1, 8, and 6 in subunits A, B, H, and C, respectively (36–38). The reduction of $V_1$ (Fig. 1) decreases the quantum yield markedly, suggesting that at least changes of the secondary structure occur as a result of disulfide formation and/or interaction of tryptophan residue(s) with side chains (39).

The conformational changes were investigated further by X-ray scattering to determine whether or not they were accompanied by changes of the quaternary structure of the $V_1$ complex. The experimental solution scattering curves of the $V_1$ ATPase under nonreducing conditions and that after reduction by DTT are presented in Fig. 2. The radii of gyration $R_g$ of the oxidized and reduced $V_1$ ATPase differ noticeably: $6.20 \pm 0.06$ and $5.84 \pm 0.06$ nm, respectively, whereas the maximum dimensions $D_{\text{max}}$ of both complexes remain the same: $22.9 \pm 0.1$ nm. Comparison of the normalized forward scattering with the values obtained for a reference of bovine serum albumin yields 560 ± 15 kDa for both forms. The sedimentation coefficient in agreement with that expected from the chemical composition of the $V_1$ ATPase. This indicates that both forms of the $V_1$ ATPase are monomeric in solution. The distance distribution functions in Fig. 3 are typical for elongated particles. The fraction of larger distances is significant diminished in the $p(r)$ function upon reduction by DTT, suggesting that the particle becomes more compact in the reduced state than in the oxidized state of the enzyme.

The low resolution shapes of the two particles determined by direct methods were displayed at a resolution of 2.7 nm in Fig. 4. The search volume was a sphere of radius $r = 11$ nm, and the packing radius of the dummy atoms was $r_p = 0.625$ nm, yielding a total of 4028 atoms in the search volume. Out of these dummy atoms, 714 and 654 were ascribed to the particle in the

\[ R = \left( \frac{1}{N} \sum_{i=1}^{N} \left( I(s_i) - I_{\text{exp}}(s_i) \right)^2 \right)^{1/2} \]  

where $N$ is the number of the experimental points and $I_{\text{exp}}(s_i)$ and $\sigma(s_i)$ are the experimental intensity and its standard deviation. The “looseness” penalty term $P(X)$ ensures that the configuration $X$ yields a compact and interconnected structure, and $w > 0$ is a penalty weight selected to have significant penalty contribution at the end of the minimization. The latter is performed starting from a random configuration and using the simulated annealing algorithm (29); details of the procedure are described elsewhere (28, 30).

Prior to the shape analysis, a constant is subtracted from the experimental data to ensure that the intensity decays as $s^{-4}$ following Porod's (31) law for homogenous particles. The value of this constant is determined automatically by DAMMIN from the outer part of the curve by linear regression in coordinates $s^2 f(s)$ versus $s^2$. This procedure yields an approximation of the “shape scattering” curve (i.e., scattering because of the excluded volume of the particle filled by a constant density). Furthermore, DAMMIN allows to incorporate additional information about particle symmetry and anisometry. The $V_1$ ATPase is known to be an elongated particle possessing a quasi-3-fold symmetry (32, 33), so these restrictions were implemented as described in the program manual.

Trypsin Digestion Studies—$V_1$ ATPase was incubated at a concentration of 4 mg/ml with trypsin in a ratio of 1:250 (w/w) in 20 mM Tris/HCl (pH 8.1) and 150 mM NaCl at 30°C. Trypsin cleavage was stopped by addition of soybean inhibitor in a ratio to trypsin of 4:1 (w/w) and applied to a gel for electrophoresis.

In-gel Tryptic Protein Digestion and Mass Spectrometric Analysis—The bands, called A$_1$ and A$_2$, were cut out from the SDS-polyacrylamide gel and destained with a solution of 25 mM ammonium bicarbonate and 50% acetonitrile for 12 h. Gel bands were cut into small pieces of 1 mm$^3$, washed three times with acetonitrile, dried for 30 min in a speed vacuum concentrator and digested according to a procedure modified from Hellman et al. (34) and Roos et al. (35). For MALDI mass spectrometry, aliquots of 0.5 $\mu$l of the digested solution were applied to a target disc and allowed to air dry. Subsequently, 0.5 $\mu$l of matrix solution (1% w/v a-cyano-4-hydroxynicotinic acid in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid) was applied to the dried sample and again allowed to dry. Spectra were obtained using a Bruker Biflex III MALDI-TOF mass spectrometer. For the identification of the protein fragments, we used a program available at the website at the University of California at San Francisco, the ProFound program at the website of Rockefeller University, the PepSearch program at the website of EMBL in Heidelberg, and TagIdent, which is available on the ExPASy World Wide Web server.

RESULTS

Removing of the sulphydryl-reducing agent, mercaptoethanol, from the purified $V_1$ ATPase from $M. sexta$ dropped the enzyme activity from 1.8 to 0.6 $\mu$mol ATP hydrolyzed per milligram of protein per minute. That inhibition of ATPase activity can be abolished by the addition of DTT, increasing the activity to 2.1 $\mu$mol of ATP hydrolyzed per milligram of protein per minute.

As a preliminary to x-ray solution scattering experiments, the fluorescence emission of the oxidized and reduced state of the $V_1$ ATPase was monitored using the intrinsic tryptophan fluorescence, to determine whether or not the inhibition coincides with conformational changes in the enzyme. The $V_1$ ATPase from $M. sexta$ contains 21 tryptophans: 6, 1, 8, and 6 in subunits A, B, H, and C, respectively (36–38). The reduction of $V_1$ (Fig. 1) decreases the quantum yield markedly, suggesting that at least changes of the secondary structure occur as a result of disulphide formation and/or interaction of tryptophan residue(s) with side chains (39).

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final DAMs of the oxidized and reduced V1 complex, respectively, yielding excluded volumes of 986 and 904 nm$^3$ and radii of gyration of 6.15 and 5.79 nm for the two models, respectively. The discrepancy with respect to the experimental data after subtracting a constant is about $50.8$ in both cases, and the final fits are presented in Fig. 2.

Both low resolution structures have characteristic mushroom-like shapes with a central stalk of significant length similar to the recently published structures of the V1 ATPase from Clostridium fervidus (32) and M. sexta (33) obtained by electron microscopy and x-ray small angle scattering, respectively. Comparison of the two models in Fig. 4 indicates that the reduction of V1 ATPase makes the peripheral regions of the molecule more compact; these changes take place both in the globular head, where the major subunits A and B are located (32, 33, 40), and in the elongated stalk. At the top of the oxidized V1 complex a crown-like structure can be observed (Fig. 4, left panel), which is similar to the top domain of the quaternary structure of this complex that was determined from solution scattering data (40) using the ab initio shape determination procedure, SASHA (simulated annealing algorithm). In the reduced form this crown has an arrow-like shape. A prominent feature of the oxidized V1 ATPase is the approximately 3.5-nm wide protuberance at the bottom of the stalk. A similar protuberance is observed at the same site of the central stalk in electron micrographs of the V1 molecule from C. fervidus (32). An intriguing observation is the conformational change of this protuberance into a wedge-like feature at the bottom of the stalk, because of reduction of the V1 molecule (Fig. 4, right panel). In summary, the V1 ATPase has an elongated mushroom-like shape with a head piece of approximately 15 and 16 nm in diameter in the oxidized and reduced V1 ATPase, respectively, and a stalk that is about 11.5 nm long.

The information content in solution scattering data is usually related to the number of Shannon channels (41, 42) in the experimental range. In our case this number is equal to $N_s = s_{max} D_{max}/\pi = 15.4$, and the use of thousands of spheres...
for *ab initio* shape retrieval might raise fears that the data would be over-interpreted. The shape of the oxidized V₁ ATPase (Fig. 4, *left panel*) is very similar to the molecular envelope computed earlier from the solution scattering data using another *ab initio* shape determination procedure (40). Given that the particle envelope in the latter study was described using only 17 free parameters, it is obvious that the effective number of independent parameters in the models presented here is much smaller than the number of dummy atoms in the search volume. The model in the simulated annealing algorithm procedure was kept at low resolution by the looseness penalty, and their spatial resolution is defined solely by the range of the data fitted as 2\( \pi \rho_{\text{max}} = 2.8 \) nm. Several independent shape restorations were performed to check the stability of the solution and yielded results very similar to those presented in Fig. 4.

Firm evidence that reduction of the V₁ molecule affects its structure was also obtained from tryptic digests of the protein in the nonreduced and reduced state. The time course of proteolysis was probed by SDS-polyacrylamide gel electrophoresis (Fig. 5). Tryptic proteolysis of V₁ is characterized by a rapid cleavage of subunit B yielding two fragments, with apparent molecular masses of 25 and 34 kDa, respectively, as determined by MALDI mass spectrometry (Fig. 5 and Ref. 43). With a longer incubation time (up to 25 min) subunit B was cleaved further, thereby increasing the amount of the 25- and 34-kDa fragments. Among the stalk subunits C–I, subunit D was cleaved most rapidly, followed by subunits F, G, C, A, and H, with subunit E being cleaved the most slowly. By contrast, reduction of the V₁ ATPase by DTT causes slow cleavage of the complex; the subunits B, D, and F become remarkably stable toward the proteolytic activity of trypsin.

Many disulfide bond-containing proteins migrate faster in SDS-polyacrylamide gel electrophoresis than do their reduced counterparts, presumably because the form containing disulfide is more compact (44). Thus migration of proteins on SDS-polyacrylamide gel electrophoresis under nonreducing conditions can be used as a sensitive measure of the extent of native disulfide bond formation (45, 46). When applied onto a polyacrylamide gel (Fig. 6A), subunit A of the oxidized form (*lane 2*) migrates as a diffuse band (\( \text{A}_1 \)), which becomes more defined toward the anode (\( \text{A}_2 \)). Prolonged electrophoresis resolved \( \text{A}_1 \) and \( \text{A}_2 \) in two bands with apparent molecular masses of 64 and 62 kDa, respectively (Fig. 6B). By comparison, the reduced form of subunit A migrates as a defined band of approximately 64 kDa (*lane 2*), a molecular mass similar to that of \( \text{A}_1 \) (*lane 3*). The bands \( \text{A}_1 \) and \( \text{A}_2 \) were identified as subunit A by MALDI mass spectrometry (not shown). Using a variety of available software packages for mass fingerprinting, we identified 20 peptides covering 46% of the subunit A sequence. However, we were not able to monitor unequivocally the appearance of disulfide bond formation between Cys\(_{254}\) and Cys\(_{532}\). This might be due to the incompleteness of in-gel tryptic digestion of the polypeptides used for mass spectrometry, which covers only 46% of the subunit A sequence. Moreover, the molecular mass difference of 2 Da between cystine and two cysteine residues cannot be easily analyzed by MALDI mass spectrometry because of the broad isotope distribution at high carbon numbers and/or imperfect sample purity because of the partially hydrolyzed asparagine or glutamine residues (47).

Disulfide bond formation in the catalytic A subunit is predicted to lock the enzyme into a closed conformation and to prevent the enzyme sterically from adopting the open conformation (reduced form), in which the catalytic site is unfilled (11, 14). The effect of CaATP binding to the enzyme was investigated by x-ray scattering to determine whether or not it was accompanied by changes of the quaternary structure of V₁. The use of CaATP as a substrate prevents possible inhomogeneity of the electron density because of the methanol, included in the MgATP assay (48, 49). In this approach CaATP was added to the enzyme prior to removing of sulfhydryl reagents (see “Experimental Procedures”). The radius of gyration for the enzyme oxidized in the presence of CaATP was found to be 6.12 ± 0.04 nm. The scattering curve and the corresponding distance distribution function (not shown) nearly coincide with that of the oxidized enzyme (see above), indicating that the quaternary structure of the oxidized form is not altered significantly by the presence of CaATP prior to oxidation.

**DISCUSSION**

Vacular ATPases have been shown to be sensitive to the redox state of their environment (11–16). A mechanism of reversible disulfide bond formation between cysteine residues at the catalytic A subunit has been proposed to regulate the V-ATPase in *vivo* (13, 14). Cys\(_{254}\) and Cys\(_{532}\) in subunit A of the bovine clathrin-coated V-ATPase have been shown to form this disulfide bond, leading to reversible inhibition of V-ATPase activity (13). The Cys\(_{254}\) residue is located in the sequence of the phosphate-binding motif (GXXGKTV), called the P-loop, whereas Cys\(_{532}\) is located in the C-terminal part and in close proximity to the adenosine binding pocket of the nucleotide (50). These residues are conserved as cysteines in the A subunit of the V-ATPase from *M. sexta* (36). It has been predicted that a disulfide bond between both residues would lock the enzyme into a closed conformation with nucleotide (ADP) unable to get out (11, 14).

In the present studies x-ray solution scattering was used to investigate the quaternary structure and subunit rearrangement of the V₁ ATPase from *M. sexta* because of redox modulation. The low resolution structure of the oxidized and reduced V₁ ATPase, derived from experimental data, display a characteristic mushroom-like shape, with a head piece containing the nucleotide-binding subunits A and B (32, 33, 40) and a stalk that accounts for the linking of catalytic site events in the head piece with ion pumping events through the V₀ complex (Fig. 4 and Refs. 32 and 40). Reduction of the oxidized V₁ complex causes remarkable conformational changes in the shape of the V₁ molecule, particularly in the crown-like region at the very top of the molecule and at the bottom region of the stalk. Based on homology of the subunits A and B to the related F-ATPase subunits β and α, respectively (50), whose N termini form a

![Fig. 4. Low resolution models of the oxidized V₁ ATPase (*left panel*) and reduced V₁ ATPase (*right panel*). The radius of the sphere is 0.625 nm. The models were displayed on a SUN Workstation using the program ASSA (60).](image-url)
b-barrel domain in a crown-like fashion (51, 52), the conformational changes at the top of the V1 ATPase presumably are due to rearrangements in the N termini of the A and B subunits. In agreement with this assignment are the two-dimensional average images of the V1VO ATPase from bovine clathrin-coated vesicles, where elongated features, attributed at least in part to the N termini of subunit A, can be seen (53). As shown more recently by three-dimensional reconstruction of the related F1F0 ATPase from Escherichia coli, a crown-like shape, which was not present in the absence of the nucleotide, evolves upon binding of the noncleavable nucleotide analogue AMP-PNP to the catalytic b subunit (54). Rearrangements in the N-terminal domains of the α and β subunits at the very top of F1 ATPase have been implicated in this structural alteration of the crown (54). Moreover, when AMP-PNP or ADP is bound to its catalytic site, subunit β adopts to the closed conformation, in which the adenine-binding pocket moves into close proximity of the P-loop and away when the binding site is empty (open conformation), as shown by the crystallographic models of the αββ γ subcomplex of bovine heart (51) and rat liver (55) F1 ATPase. There is a striking similarity in the fact that the crown structure of the E. coli F1F0 ATPase (54) evolves after binding of AMP-PNP (closed conformation), whereas a crown-like feature can be observed in the oxidized V1 ATPase, a state, in which the catalytic A subunit is proposed to be in a closed conformation (11, 14), and turns into a wedge-like shape after reduction of V1. Consistent with these features are the similarity of the radii of gyration and the distance distribution functions of the oxidized form and the V1 ATPase that has been oxidized in the presence of CaATP.

The conformational changes in the head piece because of reduction of the enzyme are in line with structural alterations in the stalk region (Fig. 4), which can be expected when the stalk couples catalytic site events with ion conduction in the VO portion. In particular the protuberance at the bottom of the stalk alters into a wedge-like shape after reduction and could enable the enzyme to transmit the activation events in the head piece to the VO complex. A model has been proposed in which V1 becomes released from VO by oxidation (15). In this model, oxidizing agents like nitrate inhibit the V-ATPase by promoting the formation of a disulfide bond, followed by the release of V1. Whether the structural changes of the protuberance observed in the present studies cause dissociation of the V1 complex cannot be resolved at this stage, although the broadening of the base of the stalk suggests a change in binding strength.

Strong evidence for significant changes in the V1 ATPase after reduction are provided from the reactivity of ATPase activity, the marked decrease of intrinsic fluorescence and protection from trypic digestion of the enzyme. Subunits B and D, shown to be an exposed stalk subunit (43), and subunit F, predicted to be at the bottom of the stalk (43, 53, 56, 57), are less accessible to trypsin in the reduced form of V1. Subunits A,
B, C, E, and F of the \textit{M. sexta} \(V_1\) ATPase have cysteine residues that are possible candidates to form disulfide bonds and might therefore cause conformational changes in this complex. However, only the nucleotide binding subunits A and B contain three and one cysteine residue(s), respectively (15, 58). Therefore, it is of interest that subunit A in the oxidized \(V_1\) complex appears in two forms (A\(_1\) and A\(_2\)), as shown by gel electrophoresis, implying that not all three A subunits have to be in an inhibited (closed) conformation.

In summary, large structural changes because of redox modulation were found by small angle x-ray scattering. Together with biochemical evidence, the x-ray scattering data indicate that upon reduction, the \(V_1\) ATPase changes its shape by altering the crown-like structure at the top and the protuberance at the bottom of the molecule into a wedge-like feature, respectively. The results are consistent with previous observations, suggesting that the V-ATPase may be inactivated in a less reducing environment (9, 10, 12, 15–17) and that this inactivation involves the formation of disulfide bonds in subunit A (13).

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