Modulation of the Oligomerization State of the Bovine F$_1$-ATPase Inhibitor Protein, IF$_1$, by pH

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Bovine IF$_1$, a basic protein of 84 amino acids, is involved in the regulation of the catalytic activity of the F$_1$ domain of ATP synthase. At pH 6.5, but not at basic pH values, it inhibits the ATP hydrolyase activity of the enzyme. The oligomeric state of bovine IF$_1$ has been investigated at various pH values by sedimentation equilibrium analytical ultracentrifugation and by covalent cross-linking. Both techniques confirm that the protein forms a tetramer at pH 8, and below pH 6.5, the protein is predominantly dimeric. By covalent cross-linking, it has been found that at pH 8.0 the fragment of IF$_1$ consisting of residues 44–84 forms a dimer, whereas the fragment from residues 32–84 is tetrameric. Therefore, some or all of the residues between positions 32 and 43 are necessary for tetramer formation and are involved in the pH-sensitive interconversion between dimer and tetramer. One important residue in the interconversion is histidine 49. Mutation of this residue to lysine abolishes the pH-dependent activation-inactivation, and the mutant protein is active and dimeric at all pH values investigated. It is likely from NMR studies that the inhibitor protein dimerizes by forming an antiparallel $\alpha$-helical coiled-coil over its C-terminal region and that at high pH values, where the protein is tetrameric, the inhibitory regions are masked. The mutation of histidine 49 to lysine is predicted to abolish coiled-coil formation over residues 32–43 preventing interaction between two dimers, forcing the equilibrium toward the dimeric state, thereby freeing the N-terminal inhibitory regions and allowing them to interact with F$_1$.

The activity of the proton-pumping ATP synthase (F$_1$F$_0$-ATPase) found in mitochondria is regulated in vivo by ADP, a proton motive force ($\Delta$pH$^+$) and by a natural protein inhibitor, IF$_1$ (1). Decrease of $\Delta$pH$^+$ can reverse the action of ATP synthase, making it hydrolyze ATP and pump protons out of the organelle. ATP hydrolysis is inhibited by the inhibitor protein, IF$_1$, which binds to ATP synthase in a 1:1 stoichiometry (2). Binding depends on the presence of MgATP. Restoration of a proton motive force favoring ATP synthesis displaces IF$_1$ from its inhibitory site and releases bound ATP, indicating that IF$_1$ prevents product release (3, 4). The binding of IF$_1$ to ATP synthase is dependent on pH. Upon reduction of pH below neutrality, the inhibitory capacity of IF$_1$ increases (5). In respiring mitochondria, the pH is relatively low outside and relatively high inside the matrix where ATP is made. In the absence of oxygen or in the presence of an uncoupler of oxidative phosphorylation, ATP synthase is poised in the direction of ATP hydrolysis, and glycolysis becomes the only source of cellular ATP. The high rate of glycolysis results in reduction of cytosolic pH (6) which is transmitted to the mitochondrial matrix, promoting inhibition of ATP hydrolysis by IF$_1$ in order to preserve ATP.

Bovine IF$_1$ is a basic protein 84 amino acids in length (7), and homologues have been characterized in mitochondria from rats (8), Saccharomyces cerevisiae (9), and plants (10). Its primary sequence is well conserved, particularly in residues 14–47 (bovine numbering) which have been defined as the minimal inhibitory sequence (11, 12). At least some of the inhibitory domain lies in residues 9–22, and its C-terminal region (approximately residues 39–84) forms an intermolecular $\alpha$-helical coiled-coil, possibly leading to the formation of dimers and higher oligomers (11). By solution NMR studies of the fragment 44–84, it has been shown that dimerization is due to the formation of an anti-parallel coiled-coil.

Although reduction of pH is necessary for IF$_1$ to bind to F$_1$, the mechanism controlling this process has been unknown until now. It has been suggested that IF$_1$ exists in two conformations as follows: an inactive, highly $\alpha$-helical, non-inhibitory form above pH 6.7, and an active, less $\alpha$-helical, inhibitory form at values below pH 6.7. Bovine IF$_1$ contains five histidine residues that are expected to have $p\text{K}_a$ values within the regulatory range of inhibitor action, and therefore, the protonation and deprotonation of these residues have been proposed to modify the structure of IF$_1$ allowing it to interact with F$_1$. In support, the mutation of histidine 49 to lysine in bovine IF$_1$ (referred to as IF$_1$-H49K) eliminates its pH sensitivity (14).

As described below, the oligomeric state of bovine IF$_1$ has been investigated by sedimentation equilibrium analytical ultracentrifugation and by covalent cross-linking. Both techniques show that IF$_1$ forms active dimers and inactive tetramers and that the oligomerization state is dependent on pH. A 12-residue segment from 32 to 43 contains amino acids that are involved in this pH-dependent interconversion between the dimer and tetramer.

MATERIALS AND METHODS

Analytical Methods—Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce) or by acid hydrolysis and amino acid analysis. SDS-PAGE$^2$ gels containing a 12–22% (w/v) acrylamide gradient separating gel and 4% (w/v) stacking gel (acylamide:
N,N-methylenebisacrylamide, 30:0.8, w/w) were cast in 10 cm × 10 cm × 0.6 mm format and run in the buffer system of Laemmli (15). Bands were stained for 15 min in Gel Stain (50% v/v) methanol, 7% (v/v) acetic acid, 0.2% (w/v) Page Blue 83. Samples of proteins for analytical ultracentrifugation or for the cross-linking experiments were dialyzed overnight against the required buffer (2.5 liters) in a cellulose membrane tubing with a molecular weight cut-off of 2 kDa (Spectrum Laboratories, Inc. Haverhill, UK).

Construction of Bacterial Expression Plasmids—The expression plasmid pRK1, contains the coding sequence for the entire bovine IF1 (11). Plasmids expressing IF1, H49K and IF1, truncated at the N terminus by 31 residues (residues 32–84) were made from pRK1, by polymerase chain reaction using the oligonucleotide primers GAA GAA ACA CAA GGA AAA TTA GAT and TAG GAA TTC CAT ATG GCA TAC TTC CGA GCT CGT GCT AAA, respectively. The plasmid encoding the fragment IF1 (residues 44–84) was described earlier (11).

Overexpression and Purification of the Bovine IF1, and Related Proteins—The overexpression of IF1, and related proteins was carried out as described previously (11) except that the host cell was Escherichia coli C41 (16). After addition of isopropyl-β-d-thiogalactopyranoside, the cells were grown overnight at 25 °C. The bacteria were centrifuged (7000 × g, 15 min, 4 °C) and resuspended in TEP buffer (150 ml), containing of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.001% (w/v) phenylmethylsulfonyl fluoride. One tablet of a protease inhibitor mixture (Roche Molecular Biochemicals, GmbH, Germany) was added at this step.

Protein purifications were carried out at 4 °C. Bacterial cells containing the various proteins were broken by being passed twice through a French pressure cell. The proteins were soluble in the cytoplasmic fraction that was clarified by high speed centrifugation (142,000 × g, 1 h), except for IF1 (residues 32–84) which formed inclusion bodies (collected by centrifugation at 45,000 × g, 40 min). The broken cell supernatants containing the proteins were dialyzed twice against TEP buffer (4 liters) using a membrane with a molecular mass cut-off of 3.5 kDa and then applied to a prepacked column of S-Sepharose HP obtained from Amersham Pharmacia Biotech equilibrated with the same buffer. IF1, IF1-H49K, and IF1, residues 44–84 eluted from the column at 0.35 ml on a linear gradient of 0–1 M sodium chloride in TEP buffer. The fragment IF1 (residues 32–84) was recovered from inclusion bodies by solubilization in 6 M guanidine HCl and 0.1 M MOPS, pH 7.0, centrifuged at 140,000 × g for 1 h, and the supernatant dialyzed against TEP buffer. Insoluble protein was removed by centrifugation at 80,000 × g, 30 min. The supernatant containing IF1, (residues 32–84) was applied to a S-Sepharose column equilibrated with the same buffer. IF1, IF1-H49K, and fragments of IF1 were dialyzed at 1 M sodium chloride. In some cases, pooled fractions containing the protein were dialyzed twice against TEP buffer. Insoluble protein was removed by centrifugation at 80,000 × g, 30 min. The supernatant containing IF1, (residues 32–84) was applied to a S-Sepharose column equilibrated with the same buffer. IF1, IF1-H49K, and IF1, residues 44–84 eluted also at 0.2 M. The recovery of each protein was about 80 mg per 6 liters of culture, and the proteins were stable for several months at −20 °C.

Inhibitor Assay—Activities of recombinant bovine IF1, and IF1-H49K were assayed at various pH values for the inhibition of ATPase activity of isolated, inhibitor-free F1,ATPase (17). IF1, or IF1-H49K (0.6 μg in each case) were mixed with F1,ATPase (12 μg, 32 pmol) to give a total volume of 200 μl in 10 mM MOPS-NaOH, for pH 5.5–7.0, or 10 mM Tris-HCl, for pH 7.0–8.5. The substrate MgATP (1 μl of a neutralized 200 mM solution) was added to the mixture. After 5 min of incubation at 37 °C, the ATPase activity was estimated by transferring 10 μl of the mixture to 1 ml of ATPase assay mixture at 37 °C and by measuring the increase in the absorbance of NADH at 340 nm for 5 min. Control activity was determined at each pH by prior incubation of F1, without inhibitor. The ATPase assay mixture contained 50 mM Tris-sulfate, pH 8.0, 50 mM potassium chloride, 2 mM magnesium chloride, 1 mM EDTA, pyruvate kinase (Roche Molecular Biochemicals) (20 μg/ml), lactate dehydrogenase (Roche Molecular Biochemicals) (10 μg/ml), 0.2 mM NADH, 1 mM phosphoenolpyruvate, and 2 mM MgATP.

Sedimentation Equilibrium Analysis—Sedimentation equilibrium experiments were performed in a Beckman Optima XLA analytical ultracentrifuge, using an An-60Ti rotor, with IF1, at either pH 5.0 (in 20 mM MOPS-NaOH, 0.15 M NaCl), pH 6.5 (in sodium cacodylate, 0.15 M NaCl), pH 7.0, or pH 8.0 (in sodium phosphate, ionic strength 0.1 M). All experiments were performed at 5.0 °C and at 15,000 rpm, with scanning at 230 nm. Cells were largely filled (400-μl sample), to give data over a wide range of concentration in each cell, at a variety of initial concentrations. After an initial scan, the centrifuge was over-speeded at 22,500 rpm for 6 h, to reduce the time taken to reach equilibrium (18), and then the speed was reduced and another scan taken, followed by further scans at intervals of 24 h. When successive scans were indistinguishable, the later scan was taken as being operationally at equilibrium, and scans averaging over 100 readings were taken for analysis. Subsequently, the centrifuge was overspeeded to dialyze out any macromolecular material from the meniscus, before slowing to equilibrium speed and taking a further scan to establish the effective base-line absorbance for each cell.

The apparent partial specific volume (ρ̂), used for analysis of the data, was measured from the density increment (ρ̂∕cμ), by Equation 1, where ρ is the density of a solution containing a concentration (cμ) of the macromolecular component at dialysis equilibrium with all the diffusible components, and ρμ is the solvent density. Densities for the solution and solvent were measured using an oscillating densimeter (19) (model DMA60 with a DMA602 cell; Anton Paar, Graz, Austria).

The protein concentration was measured by amino acid analysis.

Data were analyzed initially by taking overlapping sets of 41 data points to calculate the apparent weight average molecular mass (Mw,app), taken to be at the concentration of the middle point, by nonlinear regression with Equation 2 (20),

\[
M_{w,app} = \frac{d \ln(c)}{dr} \frac{2RT}{\omega(1 - \psi_\rho)}
\]

where Mw,app is the apparent weight average molecular mass; c is the concentration (as optical density) at radius r, and ω is the angular velocity (in radians/s). Plots of Mw,app against c were made, using the program PROFIT version 5.1.2 ppc (Quantum Soft, Zürich, Switzerland) and taking ωμ = 2.52 × 104 rad s\(^{-1}\).

Visual inspection of these plots allowed an appropriate model to be selected for further analysis of possible aggregation, by directly fitting the absorbance against radius data (21), using PROFIT with the Levenberg-Marquardt algorithm (with error estimates in absorbance from the recorded data), for selected models. In practice, it was found that at any given pH, IF1, was detectable only as a single oligomer, with non-ideality detectable in some cases but with no evidence for either dissociation or further association. (Clearly there must be some smaller oligomers, but these could not be detected and the dissociation constant (Kd) fell to the lower limit even when this was set far below the values measurable in the data.) Therefore, the fitting presented is entirely consistent with the existence of a single non-ideal single species. Taking Equation 3 for a non-ideal solute (20, 22) (assuming that only the second viral coefficient need be considered),

\[
\frac{1 + 2Bc_μ}{c_μ} dc_μ = \frac{M(1 - \psi_\rho)}{RT} rdr
\]

which leads to cμ = cμ0 exp(σ(r² – r³) – 2Bcμ0), (Eq. 4) where σ = (M(1 – ψ_μ)μ)/2RT, B is the molar second viral coefficient (in M⁻¹); cμ0 is the molar concentration of component 2 (i.e. the macromolecule) at radius rμ and M is the “monomer.”

This expression can be fitted to data by evaluating cμ against r and then converting to OD by Aμ = cμ0cμ. Since Equation 4 is transcendental (i.e. cμ appears on both sides), it was evaluated numerically for each radius, by fitting cμ against r, successively until the change was less than 10⁻⁵ of the final value. (The reference radius (rμ) was taken two-thirds down the column, and the reference concentration (cμ0) was evaluated here.) Fitting was carried out with parameters for error in both the base line and the value for M, with this latter value effectively correcting for error in ψ or ρμ (21) as well as B.

Residuals were plotted against radius, to assess the goodness of fit and, particularly, to see any systematic deviation rather than random error due to the data. Theoretical curves for Mw,app against cμ were also plotted onto the experimental plots, using the fitted parameters, so that the fit could be seen.

Covalent Cross-linking of IF1, and Related Proteins—Cross-linking of amino groups in IF1, IF1-H49K, and fragments of IF1, with dimethyl suberimidate was carried out essentially according to Davis and Stark (23). The samples were dissolved at a protein concentration (determined by BCA method) of about 0.5 mg/ml in 20 mM HEPES, pH 8.0, 1 mM EDTA, 0.001% phenylmethylsulfonyl fluoride and dialyzed overnight against the same buffer. Then the protein concentration was adjusted to 0.1 mg/ml by dilution with dialysis buffer, and dimethyl suberimidate (freshly dissolved at 20 mg/ml in the same buffer) was...
added to a final concentration of 1 mg/ml. The mixture was kept for 3 h at room temperature. Samples (10 ml) were removed at various times, dissolved in sample buffer, and analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Effect of pH on Inhibition of F₁-ATPase by IF₁ and IF₁-H49K—At the protein concentration used, the maximal inhibitory effect of IF₁ was at pH 6.5, with decreased effects at both higher and lower values (see Fig. 1). At pH 8.0, F₁ was 45% active. In contrast, the inhibitory effect of IF₁-H49K was clearly independent of pH confirming earlier work (14), and at pH 8.0, 98% of the activity of F₁ was inhibited. At lower concentrations of IF₁, higher activities of F₁-ATPase were observed, but at all F₁ concentrations investigated, the pattern of response of inhibition by IF₁ to pH was similar (data not shown).

Measurement of Apparent Partial Specific Volume—Density measurements were carried out at 5 °C on both the solvent (ρₒ) and solutions of IF₁, at dialysis equilibrium with this solvent, in buffers at pH values 5.0, 6.5, and 8.0. Protein concentrations were also determined by acid hydrolysis and amino acid analysis of replicate samples of each protein solution. From these values, the density increment (〈∂ρ/∂c²〉₀) for IF₁ at each pH was calculated and hence the apparent partial specific volume (f'). These values (Table I) were used for all calculations from the analytical ultracentrifuge data.

Sedimentation Equilibrium Studies—Sedimentation equilibrium experiments with IF₁ were carried out at 5 °C and pH values 5.0, 6.5, and 8.0. Typical plots of the apparent weight average molecular mass (M_w,app) against concentration (with data from several cells loaded at different concentrations superimposed), at each pH, are shown in Fig. 2. Also shown are typical plots of the residuals (against radius) for fitting the original centrifuge data with a model for a non-ideal “monomer.” In practice, this “monomer” was taken to be a dimer of IF₁ at pH 5.0 and a tetramer of IF₁ at pH values 6.5 and 8.0, in keeping with the obvious values of M_w,app in the direct plots.

FIG. 1. Dependence of the inhibition of F₁-ATPase by IF₁ and IF₁-H49K on pH. For assay conditions, see “Materials and Methods.” ○, IF₁; □, IF₁-H49K.

FIG. 2. Sedimentation equilibrium analysis of IF₁. Analyses were performed at pH values 5.0, 6.5, and 8.0 (shown in A–C, respectively) and at ionic strength = 100 mM. Plots of M_w,app against concentration (expressed as molarity of true monomer) are shown for each pH, together with typical examples of plots of the residuals (against radius) for fitting of a model for a non-ideal monomer (i.e., a dimer or tetramer, depending upon the pH) to the experimental data. Theoretical curves corresponding to this model, with parameters derived from the fitting, are superimposed on the plots of M_w,app against concentration.

FIG. 3. Sedimentation equilibrium analysis of IF₁-H49K. Analysis and presentation of data are as in Fig. 2, except that with this mutant protein the apparent monomer was a dimer at each pH.

TABLE I

| pH | ρₒ (g/ml) | Δρ (mg/ml) | c₂ (mg/ml) | 〈∂ρ/∂c²〉₀ (mg/ml) | f' (ml/g) |
|----|-----------|------------|------------|-------------------|----------|
| 5.0| 1.002128  | 3.422      | 14.32      | 0.239             | 0.759    |
| 6.5| 1.001527  | 2.510      | 10.80      | 0.232             | 0.766    |
| 8.0| 1.000957  | 6.179      | 25.80      | 0.239             | 0.760    |
FIG. 4. Covalent cross-linking of IF1, IF1-H49K, and fragments of IF1, with dimethyl suberimidate. Samples were removed at various times (indicated in minutes above the lanes) and analyzed by SDS-PAGE. IF1, IF1-H49K, IF1 (residues 44–84), and IF1 (residues 32–84) are shown in A–D, respectively. The positions of molecular mass markers are shown on the right in kDa.

FIG. 5. Heptad repeats involved in coiled-coil formation in IF1 and IF1-H49K. The box contains the minimal inhibitory sequence (residues 14–47). Flanking residues that appear to be involved in stabilizing the complex formed between IF1 and F1F0-ATPase (residues 10–13 and 48–56) are shown in dashed boxes. Below the sequences are shown positions a–g in heptad repeats that are predicted by the program MULTICOIL (13) to be involved in formation of a-helical coiled-coils in IF1. A window of 28 and an arbitrary cut-off probability of >90% were used in calculations. In IF1-H49K, (shown by the arrow), formation of the first heptad (residues 39–45) is not predicted (shown by dashed lines).

Plots of the residuals (Fig. 2) show that such models give a good agreement to the data, with no evidence for either association or dissociation of the dominant oligomer. At both pH 5.0 and 6.5 the best fit was obtained with some non-ideality, giving estimates for $B$ of $5.4 (±1) \times 10^4$ and $2.2 (±1) \times 10^5$ m$^{-1}$, respectively (concentrations expressed as true monomer), and $B$ was fit as zero (i.e. no non-ideality) at pH 8.0. Calculated lines for $M_{w,app}$ against concentration have been drawn onto each of the experimental figures to show the fit given with the fitted parameters. Attempts were also made to fit the data with models for a monomer/dimer equilibrium (with or without non-ideality), but in every case the dissociation constant ($K_d$) fell to the limit during the fitting, even when this was set well below the smallest value that would be detectable with this optical system.

Therefore, IF1 forms a dimer at pH 5.0. At pH 6.5 dimers and tetramers are in equilibrium, and at pH 8 the predominant form is the tetramer. Moreover, dissociation into monomers is very low, and although there must be some lower oligomers present, in equilibrium with these dominant forms, they must be at such low concentrations as not to affect the apparent mass at the experimental concentrations. Similarly, aggregation of the dimer at pH 5.0 must be very slight at these concentrations, as again it does not show in the apparent mass.

In view of the change in aggregation state observed between conditions where the inhibitor is active and inactive, the aggregation state of IF1-H49K, which does not show the pH-dependent interconversion, was investigated. Similar plots to those for IF1 were obtained (Fig. 3). They were calculated assuming the same apparent partial specific volume as IF1. The protein appears to be a dimer at all three pH values, with the residuals for fitting the model of a non-ideal “monomer” again reasonably distributed around zero. However, in this case, B was fit as zero in each case, i.e. there was no evidence for any non-ideality.

Covalent Cross-Linking of IF1, IF1-H49K, and Fragments of IF1—The oligomeric states of both IF1 and IF1-H49K were examined at pH 8.0 by covalent cross-linking with dimethyl suberimidate. The products from IF1 (see Fig. 4) contain the C-terminal region of IF1, possibly by protonation of histidine residue(s). The loss of coiled-coil structure in this region prevents the interaction between the two dimers and forces the equilibrium from tetramers to dimers, thereby freeing the N-terminal inhibitory regions and making them available for interaction with F1. See text for further discussion.

Regulation of ATP Synthase—A fragment of bovine IF1 consisting of residues 44–84 forms a symmetrical dimer of antiparallel a-helical coiled-coil structure in the C-terminal region. B, at pH 8.0, two dimers associate forming a tetramer. The interaction between the two dimers involves residues in the region from 35 to 47. These residues are placed in a window of 28 and an arbitrary cut-off probability of >90% were used in calculations. In IF1-H49K, (shown by the arrow), formation of the first heptad (residues 39–45) is not predicted (shown by dashed lines).

Mechanism of Oligomerization of IF1 and Implications for Regulation of ATP Synthase—A fragment of bovine IF1 consisting of residues 44–84 forms a symmetrical dimer of antiparallel a-helical coiled-coils, and it is likely that dimerization of intact IF1 occurs in a similar way. The region in IF1 involved in the coiled-coil is predicted to extend from residues 37 to 84, whereas in IF1-H49K the coiled-coil region is predicted to be
The loss of coiled-coil structure in IF1-H49K in this region could thus prevent tetramer formation and, therefore, maintain the protein as an active dimer from pH 5.0 to pH 8.0. This conclusion is further supported by the observations that IF1-H49K, where the lysine residue will be fully protonated, is dimeric and active at all the pH values tested. The protonation-deprotonation of histidine 49 in IF1 could change the extent of coiled-coil formation thereby affecting both oligomerization and activity of the protein in a pH-dependent manner.

These results suggest a model to explain the mechanism of action of IF1 depicted in Fig. 6. At a pH of about 6.5 (Fig. 6A), IF1 forms an active dimer in which its N-terminal inhibitory domain is exposed and is capable of interacting with the F1 domain of ATP synthase. Activation is accompanied by a decrease in IF1 helicity relative to the inactive form between residues 35 and 47 which are involved in the formation of a dimer of dimers, the inactive form (Fig. 6B). This region immediately precedes histidine 49, and so it is possible that protonation of this residue leads to the loss of helicity in this area as predicted by the MULTICOIL program (see Fig. 5). The loss of the coiled-coil structure in this region would prevent the interaction between the two dimers forcing the equilibrium from tetramers to dimers, freeing the inhibitory regions to interact with F1.

An important implication of this model is that dimeric IF1 is capable of binding to two F1 domains simultaneously. Dimeric F1F0 complexes have been detected by native gel electrophoresis in mild detergent (24). This dimerization has been suggested to involve subunits e, g, and k in yeast (25). Whether it could also involve IF1 remains to be investigated.

A number of other issues surrounding the action of IF1 remain to be elucidated. One such issue is where is the binding site of dimeric IF1 in the F1 domain? Solution NMR studies are helping to unravel the structure of the dimer, and knowledge of the oligomerization process should help in the crystallization of IF1 and in the elucidation of the structure of the IF1-F1 complex.

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