Homologous and Heterologous Inhibitory Effects of ATPase Inhibitor Proteins on F-ATPases*

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In Saccharomyces cerevisiae, at least three proteins (IF1, STF1, and STF2) appear to be involved in the regulation of ATP synthase. Both IF1 and STF1 inhibit F1, whereas the proposed function for STF2 is to facilitate the binding of IF1 and STF1 to F1. The oligomerization properties of yeast IF1 and STF1 have been investigated by sedimentation equilibrium analytical ultracentrifugation and by covalent cross-linking. Both techniques confirm that IF1 and STF1 oligomerize in opposite directions in relation to pH, suggesting that both proteins might regulate yeast F1F0-ATPase under different conditions. Their effects on bovine F-ATPases are also described. Whereas bovine IF1 inhibits yeast F1-ATPase even better than yeast IF1 or STF1, the capability of yeast IF1 to inhibit the bovine enzyme is very low and decreases with time. Such an effect is also observed in the study of the homologous inhibition of yeast F1-ATPase. Yeast inhibitors are not as effective as their bovine counterpart, and the complex seems to dissociate gradually.

The F1F0-ATP synthase complex plays a central role in energy transformation in most living organisms. In mitochondria, the synthesis of ATP requires an electrochemical proton gradient across the inner membrane to drive protons back into the matrix through the membrane domain of the F1F0-ATPase, releasing energy, which is coupled to ATP synthesis. When a cell is deprived of oxygen, its electrochemical gradient collapses, and the enzyme switches from ATP synthesis to ATP hydrolysis. In mitochondria, this hydrolytic activity is thought to be regulated by the natural inhibitor protein, IF1. The binding of IF1 to ATP synthase depends on the pH value, and below neutrality, its inhibitory capacity increases (1). Bovine IF1 is a basic protein 84 amino acids in length (2), and it has two oligomeric states, tetramer and dimer, favored by pH values above and below 6.5, respectively (3). Dimerization of bovine IF1, the active form of the protein, occurs by formation of an antiparallel α-helical coiled-coil between the C-terminal regions of monomers (4, 5). This arrangement places the inhibitory regions at distal ends of the dimer, allowing the active form to bind two F1 domains simultaneously (6). The structure also reveals that at high pH values, dimers associate into tetramers and higher oligomers via coiled-coil interactions in the N-terminal and inhibitory regions, preventing IF1 from binding to ATP synthase.

Homologues of bovine IF1 have been characterized in mitochondria from rats (7), Saccharomyces cerevisiae (8), and plants (9). Their primary sequences are well conserved, particularly over residues 14–47 (bovine numbering), which have been defined as the minimal inhibitory sequence (10). In S. cerevisiae, at least three proteins appear to be involved in the regulation of ATP synthase, namely, IF1, STF1, and STF2. Both IF1 and STF1 protein bind to F1, whereas STF2 binds to the F0 domain, and it has no inhibitory activity itself (11). Its proposed function is to facilitate the binding of IF1 and STF1 to F1 (12). Because the oligomerization state of bovine IF1 determines the activity of the protein, the oligomerization properties of the yeast proteins have been investigated by sedimentation equilibrium analytical ultracentrifugation and by covalent cross-linking. Both techniques show that IF1 and STF1 oligomerize in opposite directions in relation to pH. Their effects on F-ATPases from other species are also described.

MATERIALS AND METHODS

Analytical Methods—Protein concentrations were determined by the bicinchoninic acid method (Pierce) or by acid hydrolysis and amino acid analysis. The molecular masses of yeast IF1 and STF1 proteins were verified by electrospray ionization mass spectrometry using a Sciex API III+ triple quadrupole mass spectrometer. The folding of proteins was assessed from their two-dimensional (1H,1H) nuclear Overhauser enhancement spectroscopy NMR spectra recorded with Bruker AMX 500 and DMX 600 spectrometers. Samples of proteins for analytical ultracentrifugation were dialyzed overnight against the required buffer (2 liters) in a cellulose membrane tubing with a molecular mass cutoff of 2 kDa (Spectrum Laboratories, Inc., Haverhill, UK).

Construction of Bacterial Expression Plasmids—The expression plasmid pRKIF1 contains the coding sequence for the entire bovine IF1 (10). Plasmids expressing STF1 and yeast IF1 were made by amplification of genomic DNA from S. cerevisiae by PCR. For the IF1 gene, the forward primer TAG-GAA-TTC-CAT-ATG-CCA-GGT-TCA-TCA-CCA-TGA-GCA and the reverse primer CGA-AAG-CTT-TTC-GAT-CG-GAT-GTC-ATT-TTT-GGT-CAT-CGA-GTC-AAT-ATT were used. The forward primer TAG-GAA-TTC-CAT-ATG-TCA-GAT-GGT-CCT-CTC-GGT-GGT and the reverse primer CGA-AAG-CTT-TCA-CTT-CCA-GTA-GTT-AAT-TTT-GTT were used for the STF1 gene. The PCR fragments were digested with NdeI and HindIII and ligated into pMW172 (13).

Overexpression and Purification of the Bovine and Yeast Inhibitors—The overexpression of bovine IF1 and the related yeast proteins was carried out as described previously (3). Bovine and yeast IF1 and STF1 were eluted from a S-Sepharose column at 0.35 M NaCl in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 0.001% (w/v) phenylmethylsulfonyl fluoride). In all cases, pooled fractions containing the protein were dialyzed against TE buffer again and applied to a Q-Sepharose HP column (Amersham Biosciences) equilibrated with the same buffer. Whereas
bovine IF1 emerged on a linear gradient of 0–1 m sodium chloride at 0.4–0.5 M yeast IF1 and STF1, did not bind to the column, and both proteins were recovered in the breakthrough. The recovery of bovine and yeast IF1 proteins was about 80 mg per 6 liters of culture. However, STF1 was overexpressed relatively poorly, and about 20 mg of pure protein was recovered per 6 liters of culture.

Sedimentation Equilibrium Analysis—Sedimentation equilibrium experiments were performed in a Beckman Optima XLA analytical ultracentrifuge, using an An-60Ti rotor, with the proteins at either pH 5.0 (in 20 mM sodium cacodylate, 0.15 M NaCl), pH 6.5 (in sodium phosphate, ionic strength 0.1 M), or pH 8.0 (in sodium phosphate, ionic strength 0.1 M). All experiments were performed at 5.0 °C and at speeds between 18,000 and 21,500 rpm, with scanning at 230 nm. Cells were filled almost completely (400-µl sample) to give data over a wide range of concentrations in each cell, at a variety of initial concentrations. After an initial scan, the centrifuge was overspeeded at 1.5× the final speed for 6 h to reduce the time taken to reach equilibrium (14), and then the speed was reduced, and another scan was taken, followed by additional 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 sediment the macromolecule away from the meniscus, before slowing to equilibrium speed and taking an additional scan to establish the effective base-line absorbance for each cell.

The apparent partial specific volume (Vp), used for analysis of the data for both proteins, was measured from the density increment (dp/ρcρc), (15) for the yeast STF1 protein, at pH 5.0, by Equation 1,

\[ \frac{dp}{ρcρc} = (1 - ϕρs) \]  

(Eq. 1)

where ρ is the density of the solution containing a concentration (cρ) of the macromolecular component at dialysis equilibrium with all the diffusible components, and ρs is the solvent density. densities for the solution and solvent were measured using an oscillating densitometer (16) (model DMA60 with a DMA602 cell; Anton Paar, Graz, Austria). The protein concentration was determined by amino acid analysis.

Data were analyzed initially by taking overlapping sets of 41 datum points to calculate the apparent weight average molecular mass (Mapp), taken to be at the concentration of the middle point, by nonlinear regression with Equation 2 (15),

\[ M_{app} = \frac{dn(c)}{dc} = \frac{2RT}{ω(1 - ϕρs)} \]  

(Eq. 2)

where c is the concentration (as optical density) at radius r, and ω is the angular velocity (in radians/s), and plots of Mapp against c were made, using the program Profit version 5.1.2 pp (Quantum Soft, Zürich, Switzerland). Concentrations were calculated from the optical densities using the appropriate molar extinction coefficient for a protein molecule (and it is assumed that for the dimer will be twice this), and δA is the base-line absorbance.

The absorbance (Aρ) at every radius (r) was then calculated from Equation 5.

\[ Aρ = A0 + \left(1 + \frac{2cρexp(ωρ2 - r2)}{Kd}\right) + δA \]  

(Eq. 5)

A nonideal solute can be described by Equation 6 (15, 18) (assuming that only the second virial coefficient need be considered),

\[ Bρ = M1 - ϕρs \]  

(Eq. 6)

where B is the molar second virial coefficient (in M⁻¹), cρ is the molar concentration of the macromolecules at radius r, and M is the “monomer.” This leads to Equation 7,

\[ cρ = \frac{cρexp(ωρ2 - r2)}{cρ - c0} - 2Bρ(cρ - c0) \]  

(Eq. 7)

where cρ is the reference concentration of macromolecules at r0. This expression can be fitted to the data by evaluating cρ against r and then converting into absorbance with Equation 8,

\[ Aρ = cρ + δA \]  

(Eq. 8)

Because Equation 7 is transcendental (i.e., cρ appears on both sides), it was evaluated numerically for each radius, by fitting cρ successively until the change was less than 10⁻⁸ of the final value. (The reference radius (rρ) was taken two-thirds down the column, and the reference concentration (cρ) was evaluated here.) With both models, fitting was carried out with parameters for error in the base line, as well as for Kρ or B, respectively.

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

Covariant Cross-linking of Yeast IF1 and STF1—Cross-linking of amino groups in IF1 and STF1, with dimethyl suberimidate was carried out as described previously (3). The samples were dissolved at a protein concentration (determined by bichinchoninic acid method) of about 0.5 mg/ml in 20 mM HEPES, pH 8.0, 1 mM EDTA, and 0.001% phenylmethylsulfonyl fluoride and dialyzed overnight against the same buffer. Then, the protein concentration was adjusted to 0.15 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. After that time, samples (10 µl) were removed, dissolved in sample buffer, and analyzed by SDS-PAGE.

Inhibitor Assay—Activities of recombinant bovine and yeast IF1 and STF1, were assayed at two different pH values for the inhibition of ATPase activity of isolated bovine, yeast, and Escherichia coli F1-ATPase (19). Bovine IF1, yeast IF1, or STF1 was mixed in a 2-fold molar excess with yeast F1-ATPase (3 µg) or bovine F1-ATPase (7 µg) to give a total volume of 100 µl in a buffer of 10 mM MOPS- NaOH, pH 6.8, 1 mM EDTA, 0.001% (w/v) phenylmethylsulfonyl fluoride and 10% (v/v) glycerol. The substrate MgATP (1 µl of a neutralized 200 mM solution) was added to the mixture. After incubation at 37 °C for 5 min, the ATPase activity was estimated by transferring 10 µl of the mixture to 1 ml of ATPase assay mixture at 37 °C and measuring the decrease in the absorbance of NADH at 340 nm for 3 min. Control activity (100%) was determined by prior incubation of F1 at each pH and in the absence of any 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, 2 mM MgATP, and either 50 mM Tris-sulfate, pH 8.0, or 50 mM MOPS-NaOH, pH 6.6.

An additional assay to measure the overall extent of the activity as the amount of inorganic phosphate liberated from ATP was used. Incubation of bovine IF1, yeast IF1, or STF1, in 2-fold molar excess with yeast, bovine, and E. coli F1-ATPase, respectively, was carried out in a similar way. After 5 min at 37 °C, 10 µl of the mixture was transferred to 600 µl of either 20 mM MOPS-NaOH, pH 6.6, or 20 mM Tris-HCl, pH 8.0. The substrate MgATP (15 µl of a neutralized 200 mM solution) was added, and the mixture was incubated at 37 °C for 3 min. Then, the reaction was stopped by the addition of 400 µl of 10% SDS (w/v). A

The abbreviation used is: MOPS, 4-morpholinopropanesulfonic acid.
ferrous sulfate-ammonium molybdate solution (500 μl) was added, and the amount of inorganic phosphate was estimated by measuring the absorbance of the sample at 740 nm (20). Full activity (100%) is that of F₁ in the absence of inhibitors. Negative control activities were determined in the absence of enzyme.

RESULTS AND DISCUSSION

Characterization of Yeast IF₁ and STF₁ Proteins—Two inhibitors (IF₁ and STF₁) and at least one modulator (STF₂) seem to be involved in the regulation of F₁Fo-ATPase from S. cerevisiae. In this work, recombinant IF₁ and STF₁ have been overexpressed, purified, and characterized. The correct folding of both proteins was assessed from their two-dimensional (¹H,¹H) nuclear Overhauser enhancement spectroscopy NMR spectra. The spectra showed the proteins to be well structured and highly helical because the spectra were densely populated with

| Characterization of yeast STF₁ and IF₁ proteins | ε₂₃₀ | ε₂₈₀ | Molecular mass |
|-----------------------------------------------|------|------|---------------|
| Yeast STF₁                                   | 24,590 | 2,270 | 7,327.9       |
| Yeast IF₁                                    | 14,290 | 44.75 | 7,382.8       |

| A | B | C |
|---|---|---|
| ![Figure 1](image1.png) | ![Figure 1](image2.png) | ![Figure 1](image3.png) |

**Fig. 1. Effect of pH on the aggregation of the yeast STF₁ protein.** Plots of the apparent weight average molecular mass against total protein concentration are shown at pHs 5.0, 6.5, and 8.0 (A, B, and C, respectively). Individual calculated values from several cells are shown on the left, together with a curve calculated from the “best fit” model (see “Materials and Methods”). Examples of plots of the residuals between the measured and calculated absorbance at each radius, from the fitting of individual cells to the optimum model, are shown on the right. In practice, A was best fitted as a nonideal monomer, whereas B and C were fitted as monomer-dimer equilibria.

### TABLE I

| Characterization of yeast STF₁ and IF₁ proteins | ε₂₃₀ | ε₂₈₀ | Molecular mass |
|-----------------------------------------------|------|------|---------------|
| Yeast STF₁                                   | 24,590 | 2,270 | 7,327.9       |
| Yeast IF₁                                    | 14,290 | 44.75 | 7,382.8       |

a Molar extinction coefficients at 230 and 280 nm.

b Molecular masses in daltons.
cross-peaks including many between backbone amide signals. Their molecular masses (see Table I) were verified by mass spectrometry analysis.

The apparent partial specific volume ($\bar{\rho}$) of STF1 was measured at pH 5.0. The densities of a protein solution at dialysis equilibrium with the buffer and also the buffer were measured as 1.012003 and 1.008537 g/ml, respectively. Amino acid analysis gave a protein concentration of 1.729 mM, corresponding to 12.60 mg/ml. These values give a ($\bar{\rho}$ - $\bar{\rho}_{0}$) of 0.2751, with $\bar{\rho}_{0} = 0.7188$ ml/g. This value, together with $\bar{\rho}_{0} = 0.7188$ ml/g, was used for all calculations of the molecular mass because the two proteins are highly homologous and have similar amino acid compositions, so it was not expected that the correct value would vary significantly between the proteins or with pH.

Spectra for the proteins, together with their concentrations, gave the molar extinction coefficients shown in Table I. (It should be noted that neither protein contains any tryptophan residue and that the yeast inhibitor protein also lacks any tyrosine residues, explaining the very low molar extinction coefficients found at 280 nm.)

**Fig. 2. Effect of pH on the aggregation of the yeast IF1 protein.** Plots of the apparent weight average molecular mass against total protein concentration are shown at pHs 5.0, 6.5, and 8.0 (A, B, and C, respectively). Individual calculated values from several cells are shown on the left, together with a curve calculated from the "best fit" model (see "Materials and Methods"). Examples of plots of the residuals between the measured and calculated absorbance at each radius, from the fitting of individual cells to the optimum model, are shown on the right. In practice, all pH values were best fitted as monomer-dimer equilibria.
STF₁ proteins have been investigated at various pH values by sedimentation equilibrium analytical ultracentrifugation. Aggregation of STF₁ is affected markedly by pH. At pH 5.0, a nonideal monomer is seen, with $K_d = 450 \text{ M}^{-1}$, whereas at either pH 6.5 or 8.0, a monomer-dimer association is apparent, with $K_d = 45 \text{ or } 2 \mu\text{M}$, respectively (Fig. 1). Therefore, the protein aggregates increasingly strongly with increasing pH (i.e. the value of $K_d$ becomes lower). By contrast, yeast IF₁ shows a lesser effect of pH on the aggregation, but this is in the opposite direction, with $K_d$ increasing with pH, giving values of 1.2, 6.0, and 9.1 $\mu\text{M}$ at pH 5.0, 6.5, and 8.0, respectively (Fig. 2). Therefore, the protein aggregates less strongly with increasing pH.

The oligomeric states of both proteins were also examined at pH 8.0 by covalent cross-linking with dimethyl suberimidate (Fig. 3). The products from bovine IF₁ contain the monomer, dimer, trimer, and tetramer, as described previously (3). In contrast, only weak dimer formation was observed with yeast IF₁. Dimer and a weak trimer formation was observed for

![Fig. 3. Covalent cross-linking of yeast IF₁ and STF₁ with dimethyl suberimidate. Samples were removed after 3 h of reaction and analyzed by SDS-PAGE. Bovine IF₁, yeast IF₁, STF₁, and the combination of yeast IF₁/STF₁ are shown in lanes 1, 2, 4, and 5, respectively. Lane 3 corresponds to the molecular mass markers, with positions shown on the right in kDa.](image)

![Fig. 4. Inhibition of yeast F₁-ATPase by bovine IF₁, and yeast IF₁ and STF₁ proteins. For assay conditions, see "Materials and Methods." The control represents the activity of yeast F₁-ATPase in the absence of any inhibitor. Plots A and C represent the kinetic analysis, carried out by measuring the decrease in the absorbance of NADH at 340 nm. Histograms B and D represent the total amount of ATP hydrolyzed, calculated by measuring the amount of inorganic phosphate liberated. In A and B, the ATPase activity assay was carried out at pH 8. In C and D, the assay was carried out at pH 6.6.](image)
indicating that this protein aggregates at higher pH values (it should be noted that the protein concentrations used here are slightly higher than those used in the sedimentation equilibrium analysis). Therefore, these results are in good agreement with those obtained by analytical ultracentrifugation, showing in both cases that yeast IF, and STF, oligomerize in opposite directions. No pairwise combination between the two proteins was observed.

**Inhibition of Yeast F-ATPase**—Bovine IF, inhibits yeast F-ATPase even better than yeast IF, or STF, at both pH values tested (96% and 98% of inhibition, respectively) (Fig. 4). The standard ATPase activity assay carried out at pH 8 (Fig. 4A) shows that in contrast to the stable inhibition by the bovine inhibitor protein, the yeast IF, loses its capacity to inhibit yeast F-ATPase during the course of the reaction, becoming almost completely inactive after 3 min. The quantitative analysis shows that yeast IF, inhibits about 30% of the yeast F-ATPase activity (Fig. 4B), but the kinetic analysis (Fig. 4A) also reveals that inhibition is lost with time. STF, has almost no effect on the activity of yeast F-ATPase under the conditions employed.

As described under “Materials and Methods,” the complexes were previously incubated at pH 6.6 and then transferred to the reaction mixture at pH 8 for the activity assay. Therefore, the observed effect of the yeast inhibitor could be that the change in pH affects the binding, and therefore the complex is gradually dissociated. At pH 6.6 (Fig. 4, C and D), the activity assay shows that yeast IF, and STF, inhibit 90% and 70% of the activity of yeast F-ATPase, respectively. However, the kinetic analysis reveals that after 3 min of reaction, the capability of yeast IF, to inhibit the enzyme decreases slightly, an effect even more evident in the case of the STF, protein. Therefore, yeast inhibitors seem to be much more sensitive to pH than bovine IF, which inhibits F, at pH 8 during the 3 min of reaction, provided that the complex has been formed at a lower pH. However, it seems that even at a low pH, yeast inhibitors...
Conserved and semiconserved substitutions, respectively, according to the program CLUSTAL W (version 1.8; Ref. 23). The result was the same as that seen when the two proteins were added together at the same time (27% activity). These results showed that the inhibition system in yeast is complex and that a combination of them may be required to inhibit the enzyme fully.

Inhibition of Bovine $F_1$-ATPase—The standard ATPase activity assay carried out at pH 8 (Fig. 5A) shows that in contrast to the yeast inhibitor protein, yeast IF$_1$ fails to inhibit bovine $F_1$-ATPase. The protein loses its capacity to inhibit the enzyme after a few seconds of reaction, and, after 1 min, bovine $F_1$-ATPase activity is increased more than 100% with respect to the control. The STF$_1$ protein has no effect on bovine $F_1$-ATPase.

As described previously for yeast $F_1$-ATPase, an explanation for the observed effect of the yeast inhibitor on bovine $F_1$-ATPase could be that the change in pH affects the binding, and therefore yeast IF$_1$ is quickly released from F$_1$ under those conditions. Protein release could increase the turnover of F$_1$, increasing the activity of the enzyme to almost 120%, as shown in Fig. 5A. At pH 6.6 (Fig. 5, C and D), although the capability of yeast IF$_1$ to inhibit the bovine enzyme also decreases with time, after 3 min of reaction the protein is still able to inhibit more than 40% of the activity. Yeast IF$_1$ and STF$_1$ protein were added together to the incubation mixture, but the observed effect was the same as that of yeast inhibitor alone (data not shown). The fact that at pH 6.6 and in the presence of yeast IF$_1$, bovine F$_1$ is still 60% active, in contrast to the 10% activity found in the yeast enzyme, could reflect a specificity of binding for the inhibitor. However, bovine IF$_1$ is able to inhibit yeast $F_1$-ATPase even better than the bovine enzyme. Yeast and bovine IF$_1$ appear to compete for the binding to bovine F$_1$ because when the two proteins are added together to the incubation mixture, the activity of the enzyme decreases to 73% inhibition, in comparison with 60% and 95% inhibition with yeast and bovine IF$_1$ alone, respectively. However, an alteration in the order in which both proteins are added showed a different effect. If bovine IF$_1$ is added first, followed by yeast IF$_1$, the one bound first is not displaced by the second, and the activity is the same as that measured for the bovine inhibitor (5%). When the proteins are added in the opposite order, the result was the same as that seen when the two proteins were added together at the same time (27% activity). These results suggest that the binding of bovine IF$_1$ is more stable, and, once the complex is formed, it is not affected by the presence of the yeast inhibitor.

**Oligomerization of Yeast $F_1$ and STF$_1$ Proteins and Possible Implications for the Regulation of Yeast ATP Synthase**—In contrast to the inhibition system in yeast, only IF$_1$ appears to be required for the inhibition of bovine $F_1$-ATPase. Yeast IF$_1$, STF$_1$, and bovine IF$_1$ proteins are well conserved, especially in the region of the minimal inhibitory sequence (Fig. 6). Dimerization of bovine IF$_1$ has been shown to be crucial for the binding to bovine $F_1$-ATPase (6). The structure reveals that the inhibitory N-terminal regions are at the opposite ends of the dimer, and therefore, the protein can bind two $F_1$ domains simultaneously. Yeast inhibitors so similar in primary sequence might be expected to inhibit in a similar way. However, the work presented here reveals that the oligomerization state of these proteins is different from that of bovine IF$_1$. Neither of the yeast proteins tetramerize at high pH values in the range of concentrations used. An important issue surrounding the action of these yeast inhibitors is this: why is more than one inhibitor needed? Because the oligomerization states of yeast IF$_1$ and STF$_1$ proteins are influenced in opposite directions by pH, both proteins might regulate yeast $F_1$-ATPase under different conditions. Because the yeast IF$_1$ tends to form dimers at lower pH values and STF$_1$ tends to form dimers at somewhat higher values, IF$_1$ might inhibit $F_1$-ATPase at a lower pH, and STF$_1$ might inhibit $F_1$-ATPase at a slightly higher pH. Because the binding of the STF$_1$ protein is less stable, and the protein dissociates from $F_1$-ATPase faster than IF$_1$ (Fig. 4), this faster release might be an advantage for the enzyme to switch more quickly from ATP hydrolysis to ATP synthesis at higher pH values.

**Regulation of Bacterial ATP Synthases**—It has been proposed that in *E. coli*, the $\varepsilon$-subunit of *F$_1$F$_0$-ATPase (equivalent to the bovine $\delta$-subunit) has two conformations and may function as a “clutch” or “ratchet” to differentially regulate ATP hydrolysis and synthesis (22). It is highly improbable that the $\delta$-subunits from mitochondrial enzymes can rearrange their structure in a similar way. Neither the chloroplast nor bacterial ATPases are regulated by an inhibitor protein, and yeast and bovine IF$_1$ do not inhibit $F_1$-ATPase from *E. coli*.

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