The Dimerization Domain of the $b$ Subunit of the *Escherichia coli* F$_1$F$_0$-ATPase*

Matthew Revington, Derek T. McLachlin§, Gary S. Shaw, and Stanley D. Dunn§

From the Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1, Canada

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In this study a series of N- and/or C-terminal truncations of the cytoplasmic domain of the $b$ subunit of the *Escherichia coli* F$_1$F$_0$ ATP synthase were tested for their ability to form dimers using sedimentation equilibrium ultracentrifugation. The deletion of residues between positions 53 and 122 resulted in a strongly decreased tendency to form dimers, whereas all the polypeptides with the sequence 53–122 resulted in highly extended homodimers, whereas the deletion of residues between 103 and 110 resulted in a strongly decreased ability to form dimers using sedimentation equilibrium ultracentrifugation. The deletion of residues between positions 53 and 122 resulted in a strongly decreased tendency to form dimers, whereas all the polypeptides with the 53–122 sequence exhibited high levels of tendency to form dimers, whereas all the polypeptides with the 53–122 sequence and that the two subunits are oriented in a highly specific manner at the dimer interface.

The F$_1$F$_0$ ATP synthase is a multisubunit enzyme complex that is responsible for the production of the bulk of intracellular ATP. This complex synthesizes ATP from ADP and inorganic phosphate by utilizing a transmembrane proton gradient as an energy source. Structurally and functionally the complex can be divided into three major domains: the membrane intrinsic F$_0$ domain and the peripheral F$_1$ domain. The F$_0$ domain of the prototypical *Escherichia coli* enzyme consists of three polypeptides in the stoichiometry of $a_3b_2c_9$ (1, 2) that function in proton translocation across the inner membrane. The F$_1$ domain, which has the subunit composition of $a_3b_2$ with $\gamma_1\delta_1\epsilon_1$, performs the ATP catalytic functions. (For recent reviews of ATP synthase function and structure, see Refs. 1–3.)

Structural studies have shown that the $3\alpha$ and $3\beta$ polypeptides form a ring of alternating subunits surrounding a central region occupied by the N- and C-terminal helices of the $\gamma$ subunit (4). The remainder of $\gamma$ lies outside of the $\alpha_3\beta_2$ domain and is closely associated with the $\epsilon$ subunit (5). This $\gamma\epsilon$ subcomplex extends for 45 Å from $F_1$ to the membrane where it interacts with the $c$ subunits of the F$_0$ domain (6). Thus, $\gamma\epsilon$ forms the central “stalk” often seen in electron micrographs (7).

The binding change model (reviewed in Ref. 1) suggests that rotation of the $\gamma\epsilon$ stalk relative to the $\alpha_3\beta_2$ domain results in sequential changes in the catalytic sites on the $b$ subunit, causing them to cycle through conformations favoring substrate binding, then catalysis, and finally product release. This mechanism requires a stator structure to hold the $\alpha_3\beta_2$ hexamer in the $\gamma\epsilon$ stalk rotates within it. Recently, a structure that reaches from the membrane domain to the periphery of the catalytic domain and that is distinct from the central $\gamma\epsilon$ stalk has been observed in electron micrographs (8). This “second stalk” may function as a stator formed by the $b$ and $\delta$ subunits.

The $b$ subunit is necessary for the stable binding of $F_1$ to the membrane and for correct assembly of the complex (9). $b$ is a 156-residue polypeptide with a hydrophobic N-terminal membrane-spanning $\alpha$ helix. The remainder of the protein is highly polar and extends into the cytoplasm where it interacts with the $\delta$ subunit and the $\alpha_3\beta_2$ domain. The cytoplasmic domain of $b$, consisting of residues 24–156, has been described separately from the membrane spanning domain and forms a soluble, highly extended homodimer that can bind $F_1$ in a manner similar to the intact protein (10). Previous studies have shown that the two $b$ monomers in the complex can be covalently cross-linked without abolishing activity, confirming that the dimeric state is the functional form of the protein (11). The affinity of $b$ for $F_1$ or the isolated $\delta$ subunit is strongly correlated with its ability to form dimers (12). The only significant stretch of hydrophobic amino acids in the cytoplasmic domain occurs between Val$_{124}$ and Ala$_{132}$. Mutation of Ala$_{128}$ to asparagine has been shown to disrupt dimerization of the cytoplasmic domain (13).

Cross-linking has demonstrated that the $b-\delta$ interaction is between the C-terminal domains of each of the polypeptides (14). $\delta$ can also be cross-linked to the N-terminal region of the $\alpha$ subunit located near the top of the $\alpha_3\beta_2$ domain (15). Therefore, the second stalk is believed to be formed by the $b$ subunits extending upward from the membrane to interact with $\delta$, which binds on the top third of the $\alpha_3\beta_2$ domain. The second stalk could function as a relatively passive, rigid stator or play a more active role in catalysis by transiently storing energy in an elastic manner. Much of the cytoplasmic region of the $b$ dimer is expected to exist as a pair of extended helical rods to span the 140–150 Å from the membrane to the top of $F_1$.

In this study, we have characterized a series of N- and C-terminal truncations of the soluble $b$ domain to identify the minimal region necessary for formation of the homodimer. In addition we have made inferences about the structure of the $b$ dimer based on sites of intersubunit disulfide formation and on changes in hydrodynamic behavior upon truncation of the sequence.

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† Supported by an Ontario Graduate Scholarship.

§ To whom correspondence should be addressed. Tel.: 519-661-3055; Fax: 519-661-3173; E-mail: sdunn@julian.uwo.ca.

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Plasmid Construction—Molecular biological procedures were carried out as described by Sambrook et al. (16). Constructions were confirmed by restriction endonuclease mapping and regions of DNA derived from PCR products were sequenced to ensure that only the desired mutations had been introduced. Plasmid pDM3, which carries a synthetic sequence encoding b24–156 in pUC8, has been described previously (17). To stabilize mRNAs and enhance expression of various forms of b, a 202-base pair Bfa1-HindIII fragment of pSD100 (17) carrying the unc transcriptional terminator was inserted downstream of the b24–156 synthetic gene, using the HindIII and NdeI sites. The resulting plasmid was called pDM3T.

A number of plasmids encoding N- or C-terminal truncations of the soluble form of b were used in this work; these plasmids and polypeptides are summarized in Fig. 1. Previously, work from this laboratory described three plasmids, pSD114, pSD111, and pKK1, which express soluble b polypeptides lacking 33, 52, or 66 residues, respectively, from the N-terminal of b (17). For the current studies a series of plasmids encoding C-terminal truncations was constructed by PCR mutagenesis. In these constructions, PCR using plasmid pDM3 as a template was carried out using the M13 reverse universal primer coupled with mutagenic primers containing a HindIII restriction site and a sequence complementary to a stop codon and the desired region of synthetic b sequence. PCR products were co-digested with HindIII and a second enzyme, both unique in plasmids pDM3 or pDM3T, and inserted into one of those plasmids using the same restriction sites. Sequences encoded by the C-terminal truncations were pasted into pSD114 or pSD111 to produce forms of soluble b bearing deletions at both ends.

Another set of plasmids was constructed; each of these plasmids encoded a mutant form of b24–156 in which one of the amino acid residues between Ala103 and Glu110 of the normal sequence derived from the plasmid. For example, PAGE, polyacrylamide gel electrophoresis; BS 3, bis(sulfosuccinimidyl) carrier protein, as judged by this technique. The modifications summarized below. During purification, fractions generally similar techniques of ammonium sulfate precipitation, ion exchange chromatography, and size exclusion chromatography, with the modifications summarized below. During purification, fractions were analyzed by SDS-PAGE, and the final products were essentially pure, as judged by this technique.

**Purification of Proteins**—Induced cells expressing the polypeptide of interest were suspended in a volume of 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride equal to 10 times their monomer molecular weight, and 1 mM EDTA. The protein was loaded onto a DEAE-Sepharose column, similar to the procedure described for CM-Sepharose, similar to the procedure described for CM-Sepharose and elution with a linear gradient of 0–1 M NaCl in 25 mM sodium acetate buffer, pH 4.8. b24–122 was precipitated with 45% saturated ammonium sulfate, and run through two columns of DEAE-Sepharose, similar to the procedure described for b24–134 and b24–138. Appropriate fractions were pooled, the protein was precipitated with ammonium sulfate, and final purification was by size exclusion chromatography on a column of Sephacryl S-200.

b24–122 was precipitated with 45% saturated ammonium sulfate, dialyzed against TE buffer, and run through a column of DEAE-Sepharose, similar to the procedure described for b24–134 and b24–138. Appropriate fractions were pooled and adjusted to pH 4.8 by addition of acetic acid. Final purification was achieved by loading the sample on a column of CM-Sepharose and elution with a linear gradient of 0–1 M NaCl in 25 mM sodium acetate buffer, pH 5.0. b24–156 was precipitated with 45–70% saturated ammonium sulfate and run through two columns of DEAE-Sepharose, similar to the procedure described for b24–134 and b24–138. Appropriate fractions from the second column were pooled, and the pH was adjusted to 5.0 by the addition of acetic acid. Final purification was achieved by loading the sample onto a column of CM-Sepharose equilibrated with 30 mM sodium acetate buffer, pH 5.0, and elution with a linear gradient of 100–600 mM NaCl in 30 mM sodium acetate, pH 5.0. Protein purity was assessed by 15% SDS-PAGE gels using standard glycine running buffers except for the constructs of molecular weight less than 10 kDa (b24–122, b24–132) where improved resolution was obtained using 15% Laemmli gels (18) but with a running buffer of 100 mM Tris, 100 mM Tricine, 0.1% SDS. All chemicals and solvents used were reagent grade. Protein concentrations were determined spectrophotometrically at 280 nm except b24–156, which lacked an aromatic chromophore and was observed at 240 nm. The extinction coefficients at 280 and 240 nm were based on concentrations determined by quantitative amino acid analysis.

**Analytical Ultracentrifugation**—Purified protein samples were dialyzed into 50 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1 mM EDTA, or 50 mM sodium acetate, pH 5.0, for ultracentrifugal analyses. Initial concentrations of polypeptides were between 20 and 50 μg/ml of monomer. Unless otherwise noted all reported concentrations refer to monomer.

**Dimerization Domain of b Subunit**

The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BS 3, bis(sulfosuccinimidyl) carrier protein; NOE, nuclear Overhauser effect.

b24–156 indicates a polypeptide containing the amino acid sequence of b24–156, generally preceded by a short leader sequence encoded by the plasmid. For example, b24–116 refers to a construct consisting of residues of Tyr24 to Leu116 of the b subunit. Assuming removal of the N-terminal methionine, those constructs Bearing with Tyr24 have a leader sequence of TM1T1N5H5, whereas all other constructs used in this study have a leader sequence of SYW (Fig. 1). This step was repeated on a second column of DEAE-Sepharose to remove yet more impurities. The protein was precipitated with ammonium sulfate and finally purified by size exclusion chromatography on a column of Sephacryl S-300. b24–134 was precipitated with 35–55% saturated ammonium sulfate, dialyzed against TE buffer, and run through two columns of DEAE-Sepharose, similar to the procedure described for b24–134 and b24–138, to remove other proteins. After precipitation with ammonium sulfate, final purification was by size exclusion chromatography on a column of Sephacryl G-75.
Fujita-MacCosham model fitted for a single species. When necessary the observed values for sedimentation coefficients, $s_{20,w}$, were converted to $s_{20,w}$. Frictional coefficients ($f$) and frictional ratios ($f/f_{m}$) were calculated from $M_r$ and $s_{20,w}$ by standard methods.

NMR Relaxation Measurements—NMR spectra were collected on a Varian Unity 500 MHz spectrometer equipped with a triple resonance probe and a 2-axis pulsed field gradients. A sample of $^{13}N$-labeled b$_{24-114}$ in 50 mM acetate buffer, pH 5.0, was equilibrated at 25°C. One-dimensional T1, T2, and $^{13}N$ NOE spectra were collected using the pulse sequences described in Farrow et al. (22). The T1 relaxation was measured in an array of 25 steps from 11.2 ms to 1.36 s in 56-ms increments. The T2 signal was measured from an array of 11 spectra with relaxation delays from 16.6 to 183.6 ms in 16.6-ms steps. For the T1 and T2 data the signal envelopes were integrated and fit to determine the first order exponential decay constant. The NOE experiments were run with either a 5-s relaxation delay for the NOE base-line experiment or a relaxation delay of 2 s followed by a 3-s $^1H$ presaturation period to determine the magnitude of $^{13}N$ NOE. The ratios between the intensities of selected peaks in the NOE and no NOE experiments were calculated. Global correlation times, $\tau_w$, were calculated as described previously by Farrow et al. (22).

Chemical Cross-linking—The cross-linking of b$_{24-114}$, b$_{76-156}$, b$_{24-114}$, and b$_{34-156}$ was carried out for 10 min at room temperature using 1 mM bis(sulfosuccinimidyl)suberate (BS$^3$) (Pierce). The cross-linking reactions were conducted in the presence of 50 mM triethanolamine buffer, 1 mM EDTA, pH 7.5, and were quenched by the addition of triethanolamine-HCl, pH 7.5, to a final concentration of 100 mM. Complete quenching was achieved by leaving the reactions standing for 10 min at room temperature followed by heating in SDS sample buffer. The products of the reactions were then run on 15% SDS-PAGE gels using the Tricine running buffers previously specified.

Disulfide Bond Formation—Forms of b$_{34-156}$ bearing mutations incorporating cysteine into one of the positions between Ala$_{103}$ and Glu$_{110}$ were partially purified and tested for their ability to form disulfide bonds as described previously (17). Briefly, the partially purified proteins were reduced by dialysis into buffer containing 1 mM dithiothreitol and then dialyzed in buffer containing 0.1 M NaHCO$_3$, 10 mM CaCl$_2$, and 10 mM cysteine at 4°C to induce disulfide bond formation. After 24 h, samples were treated with 15 mM N-ethyl maleimide in SDS-PAGE sample buffer to block unreacted thiols and analyzed by nonreducing SDS-PAGE.

RESULTS

Previous and current studies of the b subunit suggest a four-domain model, which is shown in Fig. 1A to assist in the presentation of data. The N-terminal 24-residue sequence is highly hydrophobic and is embedded in the membrane with the other F$_0$ subunits. The sequence from residues 25 to 52, which is not essential for dimer formation (17), is designated the tether domain because it joins the membrane region to the beginning of the sequence essential for dimerization. The extent of the remaining sequence that is necessary for homodimer formation is defined in this report. Near the C terminus is a region that is necessary for dimerization. The sequence from residues 114 to 140 is essential for dimer formation (17). The polypeptide sequence essential for dimerization. The ex-

Expression, Purification, and Properties of C-terminal Truncations of b Subunit—Previous work from this laboratory has characterized soluble forms of b lacking the N-terminal membrane spanning domain (10), three deeper N-terminal deletions (17), and minor C-terminal truncations (17). In the current work, a series of plasmids encoding deeper C-terminal truncations of b was constructed (Fig. 1), and the polypeptides were purified and characterized. All of the new forms were extracted as soluble polypeptides, except b$_{34-114}$, which was partly soluble and partly inclusion body. The purification of these proteins by conventional techniques of ammonium sulfate precipitation, ion exchange chromatography from which the data exclude gel chromatography is described under “Experimental Procedures.”

A number of properties of these polypeptides became apparent during this work. All C-terminal deletions redissolved easily following ammonium sulfate precipitation, unlike forms with an intact C terminus. Little difference in the percentage of ammonium sulfate required for precipitation was apparent, however, until the hydrophobic region between residues 124 and 132 was removed. Forms truncated at residue 138 or earlier in the sequence failed to bind to DEAE resin at pH 8.0, underscoring the acidic nature of the C-terminal region that had been removed. There are six acidic and two basic side chains between 134 and 156; deletion of this region changes the pI for the protein from about 6 to over 8. DEAE-Sepharose was nevertheless used during the purification of these polypeptides, because it absorbed most of the other proteins in the samples. The less acidic nature of these proteins resulted in a much enhanced solubility at pH 5.0 in comparison with forms like b$_{34-156}$, which precipitate as the pH is lowered toward 5.0 (17). This property was exploited through purification on CM-Sepharose.

Determination of Oligomerization State of the b Subunit—Sedimentation equilibrium centrifugation allows accurate determination of the molecular weight of soluble proteins under native conditions and hence the stoichiometry of multimeric complexes. Previous studies of the b$_{24-114}$ construct have demonstrated that the isolated cytoplasmic domain exists primarily as a dimer (10).

In the study of the molecular weight of the b subunit truncations, sedimentation equilibrium data were fitted to a single component model to determine the average observed molecular weight, $M_{o,b}$, of the purified protein in solution. The data are presented in Table I as $M_o/M$, the ratio of $M_o$ to the molecular weight of the monomer calculated from the sequence, $M_e$. The presence of equilibrium mixtures of monomers and dimers...
The sedimentation equilibrium experiments were carried out at pH 7.5 in 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA buffer except where pH 5.0 is noted. In those studies a 50 mM sodium acetate buffer was employed.

The sedimentation equilibrium data were gathered on b53–122 at pH values between 4 and 8, using a relatively low concentration of the protein at 20°C. The highest molecular weight was observed at pH 5.0, implying that dimer formation was strongest at this pH (data not shown). The effect of temperature at pH 5.0 was then examined using the same initial concentration as in the previous experiment carried out at pH 7.5 (Table I). A higher level of thermal stability was apparent with the Mobs/Mc values at 40°C increasing from 1.25 at pH 7.5 to 1.39 at pH 5.0. Other constructs that included the C-terminal sequence beyond residue 134 precipitated at pH values lower than 6.0.

To assess the reversibility of the monomer-dimer equilibrium, each 40°C set of equilibrium data had been collected, the centrifuge was cooled to 20°C, and the protein was allowed to re-equilibrate. Subsequent data collection and analysis revealed that the Mobs/Mc ratios returned to values close to those observed at 20°C prior to raising the temperature to 40°C (data not shown). This result indicates that the observed changes were due to a reversible phenomenon rather than an irreversible process such as degradation. Further confirmation of reversibility was provided by a series of sedimentation equilibrium runs of the b24–152 construct at 35°C, a temperature at which average molecular weights indicate that both the monomeric and the dimeric states are well populated. Cells loaded with initial concentrations of 19 and 42 μM polypeptide were equilibrated at 24,000, 30,000, and 36,000 rpm, and a cell loaded with 96 μM polypeptide was equilibrated at the two lower rotor speeds. As expected for a reversible equilibrium, the average molecular weight obtained by fitting each data set

| Polypeptide             | Mobs  | Mc   | Mobs/Mc |
|------------------------|-------|------|---------|
| Cytoplasmic domain of b| 15,508| 1.87 | 1.97    |
| N-terminal truncations  |       |      |         |
| b24–156                | 14,018| 1.88 | 1.36    |
| b33–156                | 11,898| 2.02 | 1.37    |
| b24–152                | 9,863 | 1.08 | 1.10    |
| C-terminal truncations  |       |      |         |
| b24–152                | 14,933| 1.81 | 1.16    |
| b24–132                | 13,639| 1.81 | 1.29    |
| b24–122                | 11,976| 1.79 | 1.16    |
| b24–114                | 10,774| 1.29 | 1.08    |
| N- and C-terminal truncations | 8,365 | 1.96 | 1.25   |
| b53–122                | 8,365 | 1.96 | 1.25    |
| b53–122 pH 5.0         | 9,612 | 1.96 | 1.25    |

*The Mobs/Mc values reported are the average and standard error of three experiments.*
equilibrium, the relative heterodimer to homodimer populations should closely reflect those present in solution. These results reinforce the evidence that the 53–122 sequence contains all of the residues necessary for formation of dimers by the cytoplasmic domain of b.

Hydrodynamic Analyses of b Truncations—Sedimentation velocity analyses were used to study the hydrodynamic behavior of the b truncations to gain information on their shapes. Data were collected at 5 and 20 °C and corrected for the effects of density and viscosity, and the resulting sedimentation coefficients ($s_{20,w}$) and frictional ratios ($ff_{min}$) are presented in Table II. Generally, soluble forms of the b subunit exhibited $s_{20,w}$ values significantly lower than those reported for globular proteins of similar molecular weight and frictional ratios near 2 than 1. The dimer of b$_{53–156}$ measured at 20 °C can be compared (Table III) with a globular protein of nearly the same size, carbonic anhydrase, which sediments much more rapidly and has a frictional ratio just slightly higher than unity (23). These results indicate that the soluble domain of b deviates significantly from an ideal globular shape.

Sedimentation coefficients of some of the deletion constructs at 20 °C have been reported previously (14); here those data sets have been reanalyzed using the SVEDBERG program (21) that allows more accurate determinations for small proteins. Starting with the data collected at 20 °C, the N-terminal truncations from residues 24 to 53, which we call the tether domain, resulted in a modest decrease in the $s_{20,w}$ at 20 °C from 1.74 to 1.66 S, whereas the $ff_{min}$ dropped from 1.93 to 1.71, implying that this region extends from the rest of the protein. In contrast, removal of the C-terminal four residues resulted in a large decrease in the $s_{20,w}$ from 1.74 to 1.46 S and an increase in $ff_{min}$ to 2.25, implying that the C-terminal deletion produced a more extended form of the molecule. Further deletion from residues 152 to 122 (compare b$_{24–156}$ to b$_{24–122}$) did not significantly alter the sedimentation coefficient, whereas the $ff_{min}$ decreased from 2.25 to 1.95, implying deletion of an unfolded, highly extended portion of the protein. Removal of the entire C-terminal region, from residues 122 to 156 (compare b$_{24–156}$ to b$_{24–122}$ and b$_{53–156}$ to b$_{53–122}$) resulted in substantial decreases in $s_{20,w}$ but very small changes in the $ff_{min}$ values. Together, these results imply that the C-terminal domain is normally folded into a relatively compact structure at 20 °C and that this folded structure is dependent on the last four residues of the protein but independent of the tether domain.

Comparison of the properties observed at 5 °C with those at 20 °C reveals that the constructs that included an intact C terminus exhibited significantly lower $s_{20,w}$ values at 5 °C, whereas the C-terminal truncations had very similar $s_{20,w}$ values at the two temperatures. The similarity of the hydrodynamic parameters of b$_{24–156}$ at 5 °C to those of b$_{24–152}$ at either 5 or 20 °C suggests that the intact C-terminal domain is sensitive to cold-induced unfolding.

The shape of the dimerization domain by itself was investigated using the b$_{53–122}$ construct. The values obtained were not significantly different on temperature. A pH of 5.0, which would be expected to minimize the presence of monomers, gave a slightly higher sedimentation coefficient and a slightly lower frictional ratio, and these parameters probably provide the better view of the protein. In Table III we compare these values to those of myoglobin, a globular protein of nearly the same molecular weight, and to those of two coiled-coil sequences, one larger than b$_{53–122}$, and the other smaller, which have been expressed from cortxin (24). Myoglobin has a sedimentation coefficient of 2.04 S and $ff_{min}$ of 1.105 (25). In comparison, b$_{53–122}$ sediments far more slowly ($s_{20,w} = 1.36 S$), and its high

![Figure 2](image-url)
The sedimentation velocity experiments were carried out in 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5 as described under "Experimental Procedures," except when pH 5.0 is indicated. In that case the experimental buffer was 50 mM sodium acetate, pH 5.0.

### Table II

| Polypeptide                  | M (kDa) | s<sub>20,w</sub> | f/f<sub>min</sub> | τ<sub>m</sub> | Reference         |
|-----------------------------|---------|-----------------|------------------|------|-----------------|
| Cytoplasmic domain of b     | 2 x 15,508 | 1.74 | 1.93 | NA | Present work     |
| b<sub>24–156</sub>          | 30,640  | 3.30 | 1.02 | 11.4 | Reinaud et al. (23) |
| N-terminal truncations       | 2 x 8,365 | 1.36 | 1.66 | 20.4 | Present work     |
| b<sub>24–156</sub>          | 16,890  | 2.04 | 1.105 | NA | Svedberg and Pedersen (25) |
| Myoglobin (horse heart)     | 2 x 10,100 | 1.60 | 1.81 | NA | Steinmetz et al. (24) |
| Ir-4hC<sup>d</sup>           | 2 x 5,240 | 1.20 | 1.41 | NA | Steinmetz et al. (24) |
| Ir-12hH<sup>d</sup>          | 2 x 8,386 | NA | 9.1 | Grasberger et al. (26) |
| Intelexase 8 (human)        | 2 x 10,508 | NA | 9.1 | Grasberger et al. (26) |

<sup>a</sup> Molecular weights calculated from sequence or reported in the reference.  
<sup>b</sup> Frictional ratio values were calculated from the data in the present work or taken from the reference except for the cortexillin fragments. The cortexillin f/f<sub>min</sub> values were calculated from the reported molecular weight, s<sub>20,w</sub> value and an assumed partial specific volume of 0.73.  
<sup>c</sup> NA indicates that the value was not available.  
<sup>d</sup> Designations for coiled-coil fragments of cortexillin (24).

### Table III

**Comparison of b constructs with globular and coiled coil polypeptides**

| Protein                        | M (kDa) | s<sub>20,w</sub> | f/f<sub>min</sub> | τ<sub>m</sub> | Reference         |
|--------------------------------|---------|-----------------|------------------|------|-----------------|
| b<sub>24–156</sub>             | 2 x 15,508 | 1.74 | 1.93 | NA | Present work     |
| Carbonic anhydrase              | 30,640  | 3.30 | 1.02 | 11.4 | Reinaud et al. (23) |
| b<sub>53–122</sub>             | 2 x 8,365 | 1.36 | 1.66 | 20.4 | Present work     |
| Myoglobin (horse heart)        | 16,890  | 2.04 | 1.105 | NA | Svedberg and Pedersen (25) |
| Ir-4hC<sup>d</sup>              | 2 x 10,100 | 1.60 | 1.81 | NA | Steinmetz et al. (24) |
| Ir-12hH<sup>d</sup>             | 2 x 5,240 | 1.20 | 1.41 | NA | Steinmetz et al. (24) |
| Intelexase 8 (human)           | 2 x 8,386 | NA | 9.1 | Grasberger et al. (26) |

### Discussion

In the work presented here, we have studied the structure of the b subunit of ATP synthase by the use of deletions, hydrodynamic analysis, and disulfide formation between introduced cysteines. These results have given a clearer picture of the b subunit architecture, providing some surprises.

**Strength of b Dimerization—Sedimentation equilibrium**

Disulfide Bond Formation—To look for sites of interaction between the two b subunits within the dimerization domain, each residue from Ala<sup>103</sup> to Glu<sup>110</sup> was individually mutated to a cysteine in b<sub>24–156</sub> and the tendency of the resultant proteins to form intersubunit disulfide bonds was examined. The partially purified proteins were reduced with dithiothreitol and then dialyzed against buffer containing 10 mM CuCl<sub>2</sub> and 10 mM cysteine for 24 h, a technique we have used previously (17). The rationale behind this method is that Cu<sup>2+</sup>-catalyzed disulfide formation will occur between the polypeptides only if the cysteiny1 residues are close together in the dimeric structure; otherwise mixed disulfides between the cysteiny1 residue of the polypeptide and a free cysteine in solution will form. Upon analysis by nondenaturing SDS-PAGE, the interpeptide disulfide will migrate as a dimer, whereas the mixed disulfide will migrate as the monomer. In our experience with soluble forms of b, inclusion of free cysteine in the buffer is required to ensure specificity of disulfide bond formation. In the absence of free cysteine, all cysteiny1 residues tested thus far have formed disulfide bonds readily, probably because of conformational flexibility within the hydrophilic domain of the b subunit when it is not incorporated into F<sub>1</f<sub>o. As seen in Fig. 3, treatment with Cu<sup>2+</sup> in the presence of cysteine led to formation of dimers to a major extent for the protein containing the A105C mutation, to a minor extent for the I109C mutation, and possible traces of dimer can be seen for Q106C and E108C, whereas other proteins remained entirely monomeric. In additional experiments (data not shown), Western blot analysis confirmed the presence of dimeric b species for the samples indicated, and the bands were missing in samples treated with dithiothreitol, confirming that they contained disulfide linkages. These results indicate that the residue at position 105 of one b subunit must be proximal to its counterpart in the other b subunit. The other residues in this region either are not close enough or have incorrect geometry to form disulfide bonds efficiently.

The cysteine-containing b<sub>24–156</sub> proteins that did not form disulfides efficiently on their own were mixed in all possible combinations of pairs, and the experiment was repeated. No significant disulfide bond formation was observed in any case (data not shown).

**NMR Relaxation Studies**—The NMR relaxation rates of proteins in solution are dependent on overall hydrodynamic size and shape along with other effects such as chemical exchange. The global rotational correlation time, τ<sub>m</sub> of b<sub>53–122</sub> was calculated to be 20.4 ns based on 13N T1, T2, and NOE measurements, as described under “Experimental Procedures.” This τ<sub>m</sub> was considerably longer than times measured for globular proteins in the same molecular weight range (see Table III). For example, the globular and relatively compact 16.8-kDa dimer of interleukin 8 has a measured τ<sub>m</sub> of 9.1 ns (26), less than half that of b<sub>53–122</sub>. The long τ<sub>m</sub> is consistent with sedimentation velocity data in indicating a highly elongated shape.
analysis of the polar domain of b at 20 °C (10, 12, 13, 17) has generally given molecular weight values consistent with near complete formation of dimer. Nevertheless, a careful analysis of the effect of concentration on the sedimentation coefficient (27) strongly suggested that a fraction of the subunit is monomeric at low concentrations, implying a relatively weak dimerization constant. The sedimentation equilibrium studies showed that b can be converted to a largely monomeric form by simply raising the temperature to 40 °C, and the cross-linking studies confirmed that subunit exchange between different constructs occurs readily at room temperature. The monomer/dimer transition was readily reversible by a number of criteria, and the equilibrium displayed the expected concentration dependence.

The ease of thermally melting the cytoplasmic domain of b raises the question of the dimerization under growth temperatures, which are usually 37 °C but may be as high as 42 °C. It should be recognized that a concentration-dependent dynamic equilibrium exists between the monomer and dimer, and many factors outside the dimerization domain could influence this equilibrium. Among these are the membrane-embedded N-terminal domains of b (28), which would act to favor dimerization by holding the cytoplasmic domains in proximity, and interactions with δ (27) or possibly other parts of F1 or F0. For these reasons, we doubt that the cytoplasmic domains would become monomeric at physiological temperatures. However, the weak nature of the b-δ interaction implies that it may be readily influenced by stronger interactions in which the dimer may participate. Thus, it is highly possible that the b structure may change upon interaction with F1 and that it may even vary during the course of catalysis.

It should also be noted that the weak dimerization of b complicates determination of its affinity for F1 or for the δ subunit. Because these interactions depend on b in the dimeric form (12), there is no doubt that their apparent weakness (10, 27) results in part from the monomerization of b at low concentrations.

Boundaries of the Dimerization Domain—When the current project was undertaken, we expected that the C-terminal boundary of the sequence required for dimerization would fall somewhere between the end of the hydrophobic sequence V124AILAVAGA132 and the C-terminal Leu156. This expectation was based on evidence that mutations within the 124–132 region cause defective assembly of ATP synthase (13), that substitution of aspartate for Ala128 within the expressed polar domain of b produces an entirely monomeric protein (13), and that mutant forms of b with cysteines incorporated at positions 124, 128, or 132 have a strong tendency to form intersubunit disulfides (17). We were therefore surprised to discover that the protein remained dimeric following deletion of this region by truncation after residue Lys122. The studies presented here indicate that the thermal stability of the b53–122 construct was similar to that of the complete polar domain, implying that residues outside the 53–122 region contribute little to the stability of the dimer.

Heterodimer formation between b53–122 and b34–156 in the cross-linking studies confirmed the assignment of the minimal dimerization sequence by an orthogonal technique. If residues outside the b53–122 sequence contributed significantly to interactions between the subunits or if the intersubunit interactions were significantly different in b53–122 from those in the larger construct, one would expect that b34–156 would have preferentially formed homodimers. Because the observed likelihood of heterodimer formation was similar to that of homodimer formation, we conclude that the intersubunit interactions in b53–122 faithfully reflect those in the entire polar domain. At present we cannot definitively reconcile the apparent close intersubunit association in the region containing residues 124–132 with its lack of effect on dimerization.

Structure of the Dimerization Domain—Both the sedimentation coefficient and the global rotational correlation time of b53–122, determined at pH 5.0 where dimerization is nearly complete, revealed the dimerization domain to be highly extended. Under these conditions it is likely that the measured τm (20.4 ns) for b53–122 is a result of a distribution of correlation times for rotation about the long and short axes. In this case the increased magnitude of the measured τm compared with that of globular proteins of the same molecular weight should be regarded as resulting from slow tumbling about at least one axis. The frictional ratio of b53–122 shows the dimerization domain to have a degree of extension similar to that of a coiled-coil of the same molecular weight.

The existence of b53–122 as a pair of parallel helices is also supported by the finding that cysteines introduced at position 105 had a strong tendency to form disulfides, whereas a weaker tendency was exhibited by positions 106 and 109. This periodicity is consistent with that of helices and is reminiscent of the pattern reported previously for cysteines introduced in positions 124–132 (17). A significant difference, however, is that the disulfide formation at the nonoptimal positions was much lower in the 103–110 set compared with the 124–132 set, suggesting that residues within the dimerization domain are more constrained than those in the 124–132 region.

One of the cysteine mutations, Q104C, has been previously examined in the context of purified F1F0 (29). Disulfide formation could be induced by treatment with Cu2+, so it was surprising that in the present examination of the cytoplasmic domain of b by itself, disulfides formed readily with cysteine at the 105 position but not significantly at the 104 position. It would be interesting to examine the tendencies of additional positions in the 103–110 region toward disulfide formation in ATP synthase, because the pattern obtained might provide direct evidence of conformational changes in b upon F1 binding.

Domain Structure of the b Subunit—The present assignment of residues 53–122 as the dimerization domain suggests a division of the b sequence into four domains (Fig. 1A). The N-terminal 24 residues are largely hydrophobic and are essential for membrane anchoring (10). Recent studies have defined their helical structure in a solvent of chloroform/methanol/water, and the tendencies toward disulfide formation between introduced cysteine residues have suggested a dimerization interface within the membrane (28). The sequence between this membrane-spanning domain and the dimerization domain is denoted here as the tether domain. The membrane-proximal segment of this domain is relatively hydrophobic and may interact with loop regions of the a or c subunits. The highly
The dimerization domain is the enigmatic \( V^{124}A^{122} \) region, and residues nearer the C terminus, which are known to be involved in the interaction with \( \delta \) (14); here we have denoted these regions together as the \( \delta \)-binding domain. Our evidence suggests that this region has a loosely folded structure not required for dimerization that may be disrupted either by deletion of four residues from the C terminus, as suggested previously (14), or else by cooling to 5 °C. Interpretation of the sedimentation velocity results were slightly complicated by the weak dimerization of \( b \), such that a small but significant proportion of the subunit is present as monomer under the conditions of the experiment. However, it appears that this proportion is similar at 5 and 20 °C and so cannot account for the large effect. A second possible complicating factor, the higher order aggregation observed in the sedimentation equilibrium experiments at 20 °C, does not appear to be a factor during the shorter sedimentation velocity runs.\(^3\) Thus, the lower sedimentation coefficients measured at 5 °C for \( b \) constructs containing the intact C terminus can be interpreted most reasonably as a cold-induced unfolding, suggesting the importance of hydrophobic interactions in the folded structure.

Functional Implications of the Current Findings—The role of the \( b_2\delta \) stalk in the mechanism of ATP synthesis is not yet fully understood. In the structures observed in the electron micrographs, the \( b \) subunits likely form the narrow linker from the membrane to \( F_1 \) (31). The highly elongated soluble constructs analyzed in this study are consistent with that role. We have shown that the \( b \) monomer-dimer transition is dynamic and reversible and depends only on interactions between residues 53 and 122. The potential functional importance of an elastic \( b \) stalk as an energy storage device has been pointed out by Englebrecht and Junge (32); the reversibility of the dimerization interaction or conformational changes in the C-terminal domain may provide \( b_2\delta \) with elasticity.

It is also of interest to note the study of Sorgen et al. (33), who found that internal deletions of up to eleven amino acids (Ala\(^{50}\)–Ser\(^{60}\) or Leu\(^{65}\)–Ile\(^{75}\)) could be made in the \( b \) sequence without completely eliminating \( F_1F_0 \) function. All of the deletions that extended beyond residue 60 showed significant drops in activity that could only be overcome with overexpression of the mutant \( b \) subunits. On the basis of our studies, expressed cytoplasmic domains with such deletions would be expected to be monomeric. The fact that a functional \( F_1F_0 \) complex could still be obtained provided the shortened \( b \) was overexpressed implies that the dimeric state can be stabilized by either the membrane anchor, as suggested above, or else by interactions with the \( F_1 \) sector.

\(^3\) A more complete analysis of the effect of concentration on the sedimentation coefficient of \( b_{34–156} \) can be found in Fig. 3 of Ref. 27.