Formation of the *b* Subunit Dimer Is Necessary for Interaction with F_1-ATPase†

Paul L. Sorgen,‡ Michael R. Bubb,‡,§ Kimberly A. McCormick,‡,|| Arthur S. Edison,‡ and Brian D. Cain*,‡

Department of Biochemistry and Molecular Biology and Department of Medicine, University of Florida, Gainesville, Florida 32610

Received September 16, 1997; Revised Manuscript Received November 6, 1997⊗

ABSTRACT: In earlier work, we [McCormick, K. A., et al. (1993) *J. Biol. Chem. 268*, 24683−24691] observed that mutations at Ala-79 of the *b* subunit affect assembly of F_1F_0 ATP synthase. Polypeptides modeled on the soluble portion of the *b* subunit (b_{sol}) with substitutions at the position corresponding to Ala-79 have been used to investigate secondary structure and dimerization of the *b* subunit. Circular dichroism spectra and chymotrypsin digestion experiments suggested that the recombinant polypeptides with Ala-79 substitutions assumed conformations similar to the b_{sol} polypeptide. However, cross-linking studies of the Ala-79 substitution b_{sol} polypeptides revealed defects in dimerization. The efficiency of dimer formation appeared to be related to the capacity of the altered b_{sol} polypeptides for competing with F_1-ATPase for binding to F_1-depleted membrane vesicles. Ala-79 substitution polypeptides displaying limited dimerization, such as $b_{sol\ Ala-79\rightarrow Leu}$, were shown to elute with F_1-ATPase during size exclusion chromatography, suggesting a specific interaction. Sedimentation equilibrium studies indicated that 8% of the $b_{sol\ Ala-79\rightarrow Leu}$ polypeptide was in the form of a 30.6 kDa dimer and 92% a 15.3 kDa monomer. When the dimer concentration of $b_{sol\ Ala-79\rightarrow Leu}$ was normalized to the concentration of b_{sol}, both had virtually identical capacities for competing with F_1-depleted membrane vesicles for binding F_1-ATPase. The result indicated that the amount of dimer formed is directly proportional to its ability to bind F_1-ATPase. This suggests that formation of the *b* subunit dimer may be a necessary step preceding F_1-ATPase binding in the assembly of the enzyme complex.

The F_1F_0 proton-translocating adenosine triphosphate (F_1F_0 ATP) synthase is responsible for the vast majority of ATP production in *Escherichia coli* growing under aerobic conditions (*1*−*3*). The enzyme uses the energy of the electrochemical proton gradient across the plasma membrane to drive ATP synthesis. The F_1 sector consists of five dissimilar subunits (α_3, β_3, γ, δ, and ϵ) and houses the catalytic sites for ATP synthesis or hydrolysis. The F_0 sector consists of three integral membrane subunits (*a*, b_2, and c_{9-12}) and conducts proton translocation.

The high-resolution structure of the bovine F_1-ATPase supports the hypothesis that the binding change mechanism for ATP synthesis results from rotation of the γ subunit relative to the $\alpha_3\beta_3$ subunit hexamer, by indicating that each of the β subunits exists in a different conformation (*4*). Physical evidence for rotation was obtained by observation of the movement of the γ subunit relative to the three β subunits in chemical cross-linking studies (*5*), and in recovery after photobleaching experiments (*6*, *7*). Recently, direct observation of the rotation was achieved by the attachment of a fluorescent actin filament to the γ subunit (*8*).

The *b* subunit forms a dimer in the intact F_1F_0 ATP synthase (*9*). The amino-terminal hydrophobic domain of the *b* subunit is thought to form a single transmembrane-spanning domain, but most of the protein is hydrophilic and extends above the plane of the membrane extending toward F_1 (*2*). The secondary structure of the hydrophilic domain is largely α-helical. One of the primary lines of evidence for this structure comes from the expression and purification of the hydrophilic portion of the *b* subunit (b_{sol}) (*10*). The b_{sol} polypeptide displayed properties suggesting that it assumes a predominately α-helical conformation forming an elongated dimer (*10*). More importantly, the b_{sol} dimer was capable of specific interactions with the F_1-ATPase, and cryoelectron microscopy of the b_{sol}−F_1-ATPase complex showed that b_{sol} is preferentially oriented toward one of the β subunits (*10*, *11*). Clearly, the *b* subunit is central to the interaction between F_1 and F_0, but it is not yet clear whether the *b* subunit assumes a static position relative to the F_1 $\alpha_3\beta_3$ hexamer or participates in the rotational motion.

A number of *b* subunit mutations reduced steady-state levels of the F_1F_0-ATPase complex (*12*−*16*). The phenotypes of these mutants are thought to result from a structural defect in the *b* subunit resulting in a failure to productively participate in subunit interactions. Interactions with the other F_0 subunits or with F_1 are altered by specific amino acid substitutions in the *b* subunit. Apparently, an interaction between the hydrophilic domain of the *b* subunit and F_1 subunits is required for assembly of the F_1F_0 ATP synthase.

In this study, collections of mutations affecting b_{Ala-79} were transferred to the comparable position in a b_{sol} expression

† This work was supported by U.S. Public Health Service Grant GM43495 (to B.D.C.).

* To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, Box 100245, University of Florida, Gainesville, FL 32610. Fax: (352) 392-7532. E-mail: bcain@biochem.med.ufl.edu.

‡ Department of Biochemistry and Molecular Biology.

§ Department of Medicine.

|| Present address: Department of Pharmacology, University of Washington, Seattle, WA 98195.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1997.

S0006-2960(97)02309-X CCC: $15.00
Published on Web 01/01/1998

plasmid to investigate the structural failure involved with these substitutions. For clarity, the numbering of amino acids in the *b* subunit has been retained in the recombinant b_{sol} polypeptides (i.e. Ala-79 in the model polypeptide occupies the same relative position as Ala-79 of the *b* subunit). The expressed b_{sol} polypeptides were analyzed for formation of dimers and the capacity for participating in a specific interaction with F_1-ATPase. We demonstrate that replacing $b_{sol\ Ala-79}$ affects dimerization of model b_{sol} polypeptides, and that the extent of dimerization is directly proportional to the interaction of b_{sol} with F_1-ATPase. We also synthesized peptides spanning the region of Ala-79 in order to investigate the secondary structural properties by nuclear magnetic resonance (NMR)[1] spectroscopy.

EXPERIMENTAL PROCEDURES

Materials. Molecular biology enzymes were purchased from Life Technologies Inc. (Grand Island, NY) and New England BioLabs Inc. (Beverly, MA). Reagents were purchased from Sigma (St. Louis, MO), BioRad Laboratories (Hercules, CA), and Fisher Scientific (Pittsburgh, PA). The anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody (from donkey), electrochemiluminescence Western blotting detection reagents, and Hyperfilm were obtained from Amersham Life Science Inc. (Arlington Heights, IL). Polyclonal antibodies against SDS-denatured *b* subunit (*17*, *18*) were kindly provided by K. Altendorf (Univeristat Osnabruck). The b_{sol} expression plasmid pSD59 (*10*) was a generous gift from S. Dunn (University of Western Ontario). Oligonucleotides and peptides were synthesized by the core facilities of the University of Florida Interdisciplinary Center for Biotechnology Research.

Construction of Recombinant b_{sol} Plasmids. In previous work (*15*), site-directed mutagenesis was employed to generate a collection of *uncF(b)* gene mutations altering the codon for $b_{Ala-79\rightarrow Leu,Ile,Val,Lys,Gln,Glu,Pro}$. The *uncF(b)* mutations were transferred to plasmid pSD59 (b_{sol}). Plasmids pKAM44.1-44.7 ($b_{sol\ Ala-79\rightarrow Leu,Ile,Val,Lys,Gln,Glu,Pro}$) were constructed by ligation of 441 bp *Ppu*MI-*Bst*EII fragments from *uncF(b)* mutation plasmids (*15*) to the 2.0 kbp *Ppu*MI-*Bst*EII fragment of pSD59 (b_{sol}). The nucleotide sequence of each plasmid was verified, and the expression of b_{sol} polypeptides was confirmed by immunoblot analysis using the *b* subunit specific antiserum.

Purification of Recombinant b_{sol} Polypeptides. The b_{sol} polypeptides were purified essentially as described by Dunn (*10*). *E. coli* strain 1100 was transformed with b_{sol} expression plasmids pKAM44.1-44.7 and then inoculated into 500 mL of Luria Bertani medium (LB). Cultures were incubated at 37 °C with continuous agitation. At a turbidity of 75 Klett units, 1 mM isopropyl β-D-thiogalactopyranoside was added, and growth was allowed to proceed to 150 Klett units. The cells were harvested by centrifugation (10 000 rpm for 10 min), washed with TM buffer (50 mM Tris-HCl and 10 mM $MgCl_2$ at pH 7.5), and stored frozen at −20 °C.

Cells were suspended in TM buffer and disrupted by two passages through the French pressure cell at 20 000 psi. Cell debris was removed by centrifugation (12000*g*, 10 min), and the membranes were collected by high-speed centrifugation (150000*g*, 1.5 h) in a Beckman Ti-70.1 rotor. Ammonium sulfate was added dropwise to the supernatant to a final concentration of 40% of saturation. The precipitate was collected by centrifugation (40000*g*, 30 min), dissolved in 5 mL of TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8.0), and then dialyzed overnight against the same buffer.

The proteins were applied to a column of DEAE-Sepharose (1.2 × 15 cm) equilibrated with TE buffer. The b_{sol} polypeptides were eluted with a linear gradient of 0 to 250 mM NaCl in 150 mL of the same buffer (*10*). Fractions containing b_{sol} were identified by SDS−PAGE. The samples were pooled and concentrated to a volume of 2−3 mL using an Amicon Centriprep-10 membrane (Beverly, MA) and applied to a column (1.5 × 100 cm) of Sephacryl S-300 HR equilibrated in TE buffer. The protein was eluted at a flow rate of 4−5 mL/h. Fractions containing recombinant b_{sol} polypeptides were stored at −80 °C. Protein concentrations were determined by a modified Lowry procedure (*19*).

Binding to F_1-ATPase. Inverted membrane vesicles derived from *E. coli* strain 1100 were stripped of F_1-ATPase by the method of Weber et al. (*20*). F_1-ATPase was removed by suspension in ATPase release buffer [5 mM TES, 0.5 mM DTT, 0.5 mM EDTA, 0.5% 6-aminohexanoic acid (EACA), and 15% glycerol at pH 7.0]. The F_1-depleted membranes were collected by ultracentrifugation, suspended in a minimal volume of release buffer, and stored frozen at −80 °C.

Binding of b_{sol} polypeptides to F_1 was studied by competition with F_0. This was done using reconstitution experiments in which purified F_1-ATPase was incubated for 10 min at room temperature with F_1-depleted membrane vesicles in 0.1 mL of MMK buffer (50 mM MOPS, 10 mM $MgCl_2$, and 300 mM KCl at pH 7.3) in the presence or absence of recombinant b_{sol} polypeptides. Each sample was diluted to 3 mL by the addition of 2.9 mL of MMK buffer and transferred to a cuvette. Membrane energization in membrane vesicles was measured by the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (1 μg) (ACMA) and fluorescence recorded with excitation at 410 nm and emission at 490 nm (*21*). ATP was added to a final concentration of 0.4 mM.

Immunoblot Analysis. Proteins were loaded on a 15% Tris-Glycine SDS BioRad Ready Gel and electroblotted onto nitrocellulose. Antibody incubation was performed essentially as described by Tamarappoo et al. (*22*) using a 1:25000 dilution of *b* subunit specific antibodies. Secondary antibody incubation was performed with horseradish peroxidase-linked donkey anti-rabbit antibody (1:50000 dilution), and antibody binding was detected by chemiluminescence using the ECL system (Amersham Life Science Inc.). Signals were visualized on Hyperfilm-HCL using a Kodak X-OMAT instrument.

Analytical Centrifugation. Sedimentation equilibrium experiments were performed in a Beckman XLA analytical ultracentrifuge at 14 900 rpm at 4 °C in MMN buffer (10 mM MOPS, 10 mM $MgCl_2$, and 300 mM NaCl at pH 7.3). Absorption optics were employed to determine the gradient at equilibrium, with the measurement of absorption at 280 nm as a function of radius. The gradient was invariant for

[1] Abbreviations: NMR, nuclear magnetic resonance; LB, Luria Broth; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ACMA, 9-amino-6-chloro-2-methoxyacridine; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; BS^3, bis(sulfosuccinimidyl) suberate.

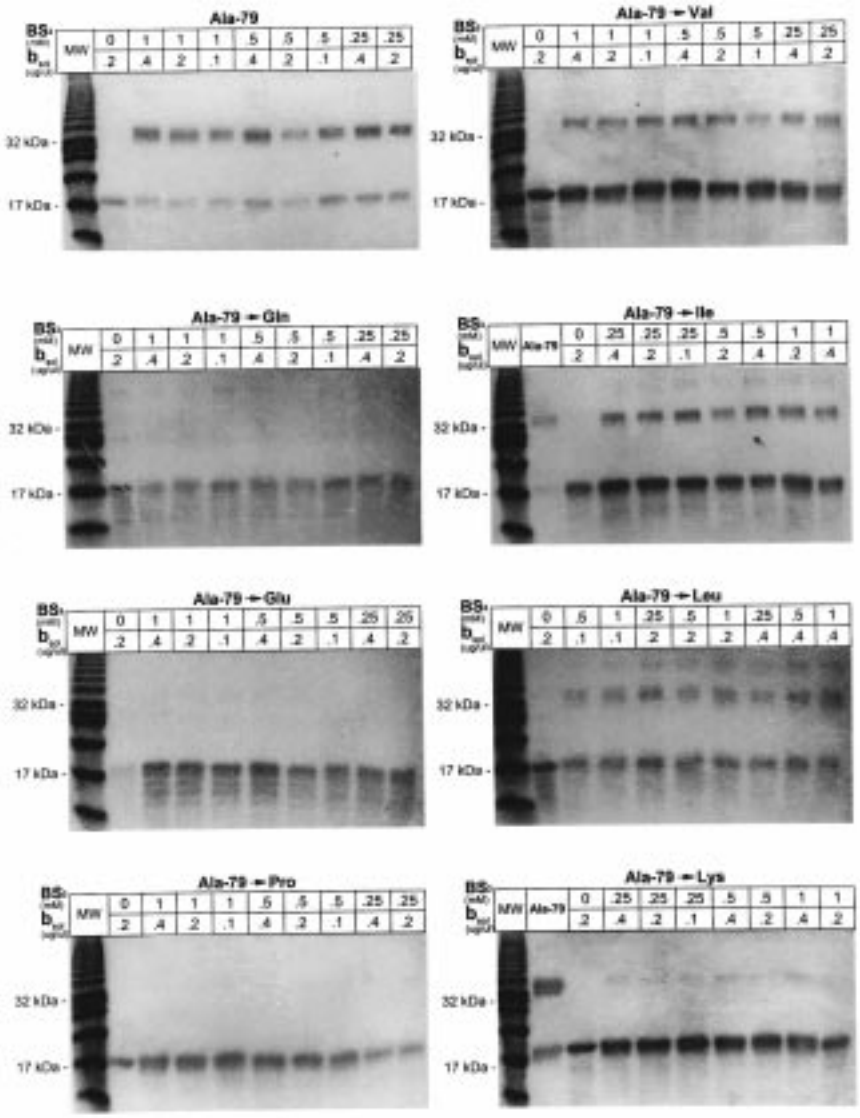

FIGURE 1: Cross-linking recombinant b_{sol} polypeptides with bis(sulfosuccinimidyl) suberate (BS^3). Recombinant b_{sol} polypeptides, at concentrations of 0.1, 0.2, and 0.4 mg/mL in TE buffer (50 mM triethanolamine and 1 mM EDTA at pH 7.5), were treated for 10 min at 25 °C with BS^3 at concentrations of 0.25, 0.5, and 1.0 mM, respectively. The reaction was stopped by addition of ethanolamine-HCL at pH 7.5 to a final concentration of 100 mM. After 10 min, samples were heated to 90 °C in SDS loading buffer for 3 min. Equal amounts of protein (1.8 μg) were run on a 15% SDS−PAGE. The recombinant b_{sol} polypeptides are indicated above each panel. The molecular mass standards for the 17 and 32 kDa bands have been labeled.

24 h after equilibrium was achieved. Data were fit simultaneously for mutant and wild-type b_{sol} assuming a homogeneous population of subunits of identical mass (i.e. the single amino acid substitution was ignored) with the extent of oligomerization for each protein varying independently. Fitting parameters included the subunit mass and the concentration of each identified oligomer for each of the individual proteins (*23*). The subunit partial specific volume of 0.741 cm^3/g (*10*) was assumed to be independent of oligomer size. The Marquard−Levenberg method was employed for nonlinear, least-squares curve fitting (*24*), and model selection was based on minimization of the sum of squares of the residuals (*25*).

Assays of F_1F_0 ATP Synthase Activity. ATP hydrolysis activity of membrane fractions was assayed by the acid molybdate method (*26*). Membranes were assayed in TM buffer (50 mM Tris-HCl and 1 mM $MgCl_2$ at pH 9.1) for determinations of linearity with respect to both time and enzyme concentration.

Nuclear Magnetic Resonance. NMR data on b_{sol} peptides were collected on a Varian Unity 600 MHz spectrometer at the University of Florida Center for Structural Biology. All peptides were dissolved in 90% H_2O/10% D_2O, and the pH for each sample was adjusted to approximately 5.5 with KOH. TSP was added for a chemical shift reference (0.0 ppm). All data were collected with a spectral width of 8000 Hz in each dimension and the ^1H carrier frequency on the water resonance, which was reduced by gradient excitation sculpting (*27*). Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) data (*28*) were collected with a mixing times of 150, 200, and 250 ms. Total correlation spectroscopy (TOCSY) data (*29*) were obtained using an MLEV-17 mixing sequence and a mixing time of 60 ms. Between 800 and 1024 indirect data points were collected using States−TPPI (*30*) for indirect quadrature detection. Data were processed using NMRPipe (*31*) by multiplying both dimensions with squared cosine functions and zero filing to 4096 points prior to Fourier transformation.

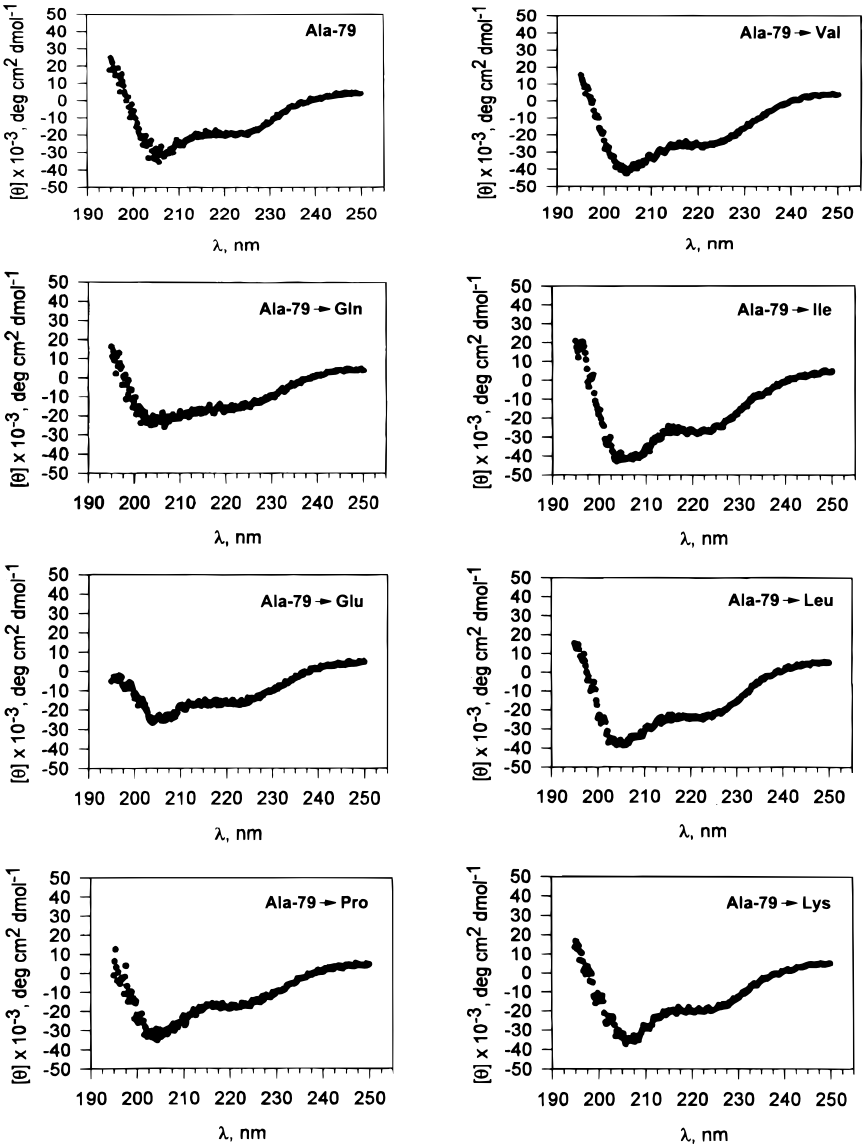

FIGURE 2: Circular dichroism spectra of recombinant b_{sol} polypeptides. Spectra of recombinant b_{sol} polypeptides were determined at 0.8 mg/mL protein using a Jasco J-500C spectrophotometer. The spectra are presented as mean residue ellipticity. The recombinant b_{sol} polypeptides are indicated on each panel.

Sequential assignments were made using TOCSY data to identify inter-residue connectivities and NOESY data to identify sequential connectivities according to standard methods (*32*). The data analysis was done manually with the computer program NMRView (*33*).

RESULTS

Preparation of Recombinant b_{sol} Polypeptides. To investigate the effects of Ala-79 substitutions on the conformation of the *b* subunit, b_{sol} expression plasmids carrying the mutations were constructed. Plasmids pKAM44.1-44.7 ($b_{sol\ Ala-79\rightarrow Leu,Ile,Val,Lys,Gln,Glu,Pro}$) directed expression of a recombinant b_{sol} polypeptide under the control of the *lac* repressor. Each of the b_{sol} polypeptides was purified to homogeneity. The yield of recombinant b_{sol} polypeptides obtained from approximately 2.0 g of cells (wet weight) was between 3 and 8 mg of pure protein.

Characterization of Recombinant b_{sol} Polypeptides. The recombinant b_{sol} polypeptides resembled wild-type b_{sol} in that they had chromatographic characteristics suggesting non-globular conformations. Chromatography through Sephacryl S-300 suggested highly extended conformations for all of the polypeptides on the basis of a mobility far in excess of that expected for a polypeptide with a deduced molecular mass of 15 459 Da (data not shown). This was probably due to the elongated structure of the b_{sol} polypeptide (*10*). It should be noted that the peak fraction for the recombinant b_{sol} polypeptides typically eluted from the column at a slightly later fraction than wild-type b_{sol}.

Since the native b_{sol} polypeptide was known to spontaneously form a dimer (*10*), one possibility for the slower mobility in gel filtration chromatography was that the Ala-79 replacement b_{sol} polypeptides favored monomeric conformations. To test this hypothesis, chemical cross-linking of the recombinant b_{sol} polypeptides was performed. Solutions of recombinant b_{sol} polypeptides were cross-linked with the irreversible agent bis(sulfosuccinimidyl) suberate (BS^3) (Figure 1). BS^3 is a homobifunctional cross-linking agent of primary amines with a span of 11.4 Å. Cross-linking was observed for the $b_{sol\ Ala-79\rightarrow Leu,Ile,Val}$ polypeptides, indicating

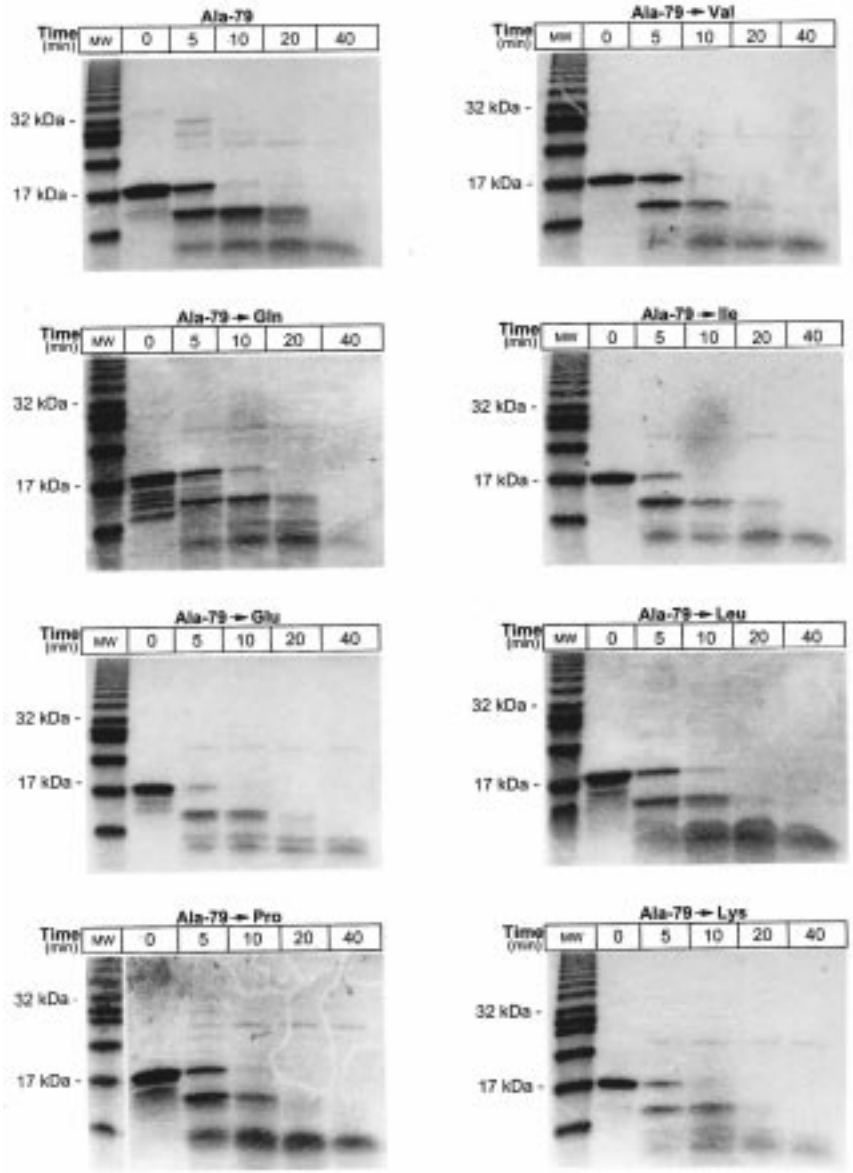

FIGURE 3: Chymotrypsin digestion of recombinant b_{sol} polypeptides. Chymotrypsin (10 μg/mL) was added to a solution of 0.5 mg/mL recombinant b_{sol} polypeptides in TE buffer (50 mM Tris-HCl and 1 mM EDTA at pH 8.0) at 25 °C. At the times indicated, aliquots were removed and the reaction was stopped by the addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM. Samples were held on ice for 10 min, treated with SDS loading buffer, and heated immediately at 90 °C. Each lane was loaded with 2 μg of protein. The recombinant b_{sol} polypeptides are indicated above each panel. The molecular mass standards for the 17 and 32 kDa bands have been labeled.

the presence of dimers. However, cross-linking was not as efficient as observed for wild-type b_{sol}. Even less cross-linked product was observed when the $b_{sol\ Ala-79\rightarrow Lys}$ was studied, and no dimers were detected in experiments involving the $b_{sol\ Ala-79\rightarrow Gln,Glu,Pro}$ polypeptides (Figure 1). The cross-linked bands had the expected mobility for dimers in SDS−PAGE, and an immunoblot experiment confirmed that the 34 kDa cross-linked product was indeed b_{sol} polypeptide (data not shown). Slower migrating material was observed in the $b_{sol\ Ala-79\rightarrow Leu,Ile}$ experiments. This was not necessarily due to the hydrophobic substitutions because no such band is seen in the $b_{sol\ Ala-79\rightarrow Val}$ studies, and Dunn (*10*) observed similar bands in earlier studies of the b_{sol} polypeptide.

The secondary structures of the Ala-79 substitution b_{sol} polypeptides were determined using circular dichroism. In every case, the far-ultraviolet spectra for all recombinant b_{sol} polypeptides showed the minima characteristic of α-helixes at 221 and 207 nm (Figure 2). This suggested that the substitutions for Ala-79 resulted in retention of largely α-helical character. However, the spectra from the $b_{sol\ Ala-79\rightarrow Gln,Glu}$ were not identical with the spectra collected using the b_{sol} polypeptide.

Hoppe et al. (*34*) showed that the *b* subunit in F_0 was cleaved rapidly by chymotrypsin at the site near the carboxyl terminus, and native b_{sol} was digested in the same manner (*10*). Treatment of the recombinant b_{sol} polypeptides with chymotrypsin also resulted in the rapid generation of a 15 kDa species whose further digestion was relatively slow (Figure 3). The rapid chymotypsin digestion on the recombinant b_{sol} polypeptides compares favorably to digestion of the *b* subunit in F_0, arguing for a conformation similar to the native *b* subunit proximal to the carboxyl termini.

Interaction with F_1-ATPase. To determine if the b_{sol} polypeptides were capable of participating in a specific

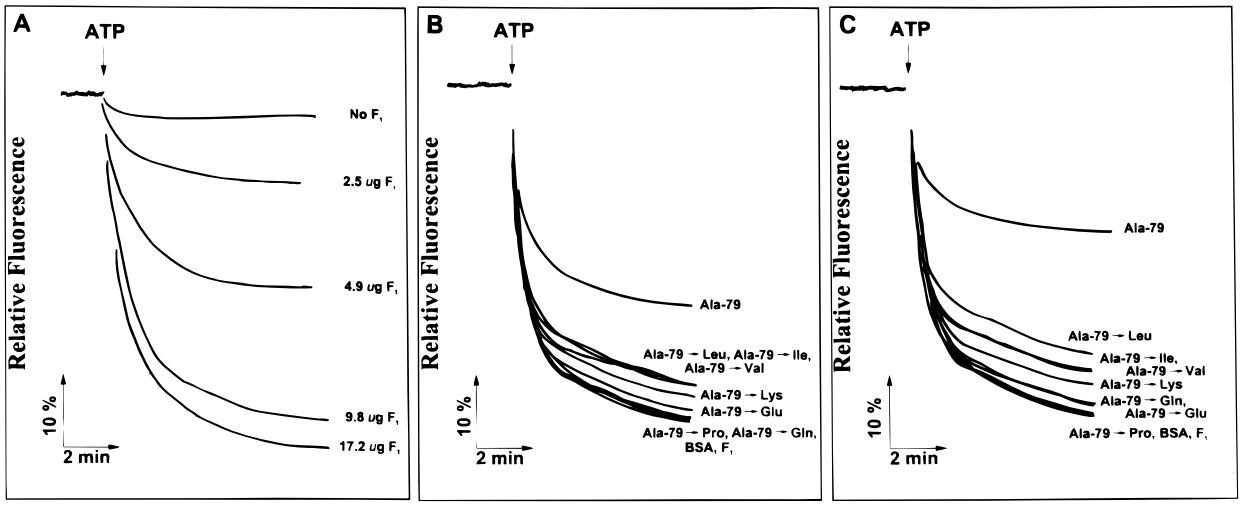

FIGURE 4: Competition of recombinant b_{sol} polypeptides for reconstitution of F_1 to F_0. Membrane energization was determined as described in Experimental Procedures. F_1-ATPase-depleted membranes (0.125 μg of protein) were reconstituted with F_1-ATPase by incubation for 10 min in 0.1 mL of MMK buffer (50 mM MOPS, 10 mM $MgCl_2$, and 300 mM KCl at pH 7.3) at 25 °C. (A) Varying concentrations of F_1-ATPase were reconstituted with depleted membranes. (B) Recombinant b_{sol} polypeptides (21 μg) were added to F_0 prior to being reconstituted with F_1-ATPase (9.8 μg). (C) Recombinant b_{sol} polypeptides (64 μg) were added to F_0 prior to reconstituted with F_1-ATPase (9.8 μg). Bovine serum albumin was used as a control for nonspecific binding.

interaction with F_1-ATPase, a series of binding studies were performed. The first series of experiments were competition assays measuring inhibition of reconstitution of F_1F_0 ATP synthase. Reconstitution of the intact enzyme was monitored by following ATP-dependent proton pumping activity using the fluorescent dye ACMA. ATP-driven energization can be restored to membrane vesicles depleted of F_1-ATPase by adding pure F_1-ATPase and allowing it to bind to the F_0 sites (Figure 4A). Maximal fluorescence quenching required about 17 μg of F_1-ATPase to reconstitute the 0.125 mg of depleted membranes present in each assay.

If the recombinant b_{sol} polypeptides interact with F_1-ATPase at the same site as the *b* subunit, then it would be expected to inhibit reconstitution by competing with F_0 for a limited number of F_1-ATPase molecules (Figure 4B,C). The addition of the $b_{sol\ Ala-79\rightarrow Pro}$ polypeptide had a negligible effect on fluorescence quenching, suggesting that this polypeptide had little affinity for F_1-ATPase. However, the $b_{sol\ Ala-79\rightarrow Leu,Ile,Val}$ polypeptides, and to a lesser extent $b_{sol\ Ala-79\rightarrow Lys,Gln,Glu}$, exhibited some inhibition of F_1 binding to F_0. Apparently, at least some of the $b_{sol\ Ala-79\rightarrow Leu,Ile,Val,Lys}$ polypeptides within the populations of molecules present assumed conformations that resembled the *b* subunit sufficiently to compete with F_0. The other Ala-79 substitutions were sufficient to virtually abrogate inhibition of F_1 association with F_0.

The interaction of recombinant b_{sol} polypeptides with F_1-ATPase was demonstrated directly by the isolation of an F_1-ATPase complex with b_{sol}. The $b_{sol\ Ala-79\rightarrow Leu}$ and $b_{sol\ Ala-79\rightarrow Pro}$ polypeptides were selected as representatives of Ala-79 substitutions allowing dimer formation and no dimerization, respectively. F_1-ATPase was mixed with excess $b_{sol\ Ala-79\rightarrow Leu}$ or $b_{sol\ Ala-79\rightarrow Pro}$, and then F_1-associated polypeptides were separated from the free by size exclusion chromatography on a Superose 12 column. The model proteins were detected by immunoblot analyses (Figure 5). The b_{sol} and $b_{sol\ Ala-79\rightarrow Leu}$ polypeptides were detected in fractions containing the peak in F_1-ATPase hydrolysis activity, 30.7 units in fraction 79. The b_{sol}, and to a much lesser extent $b_{sol\ Ala-79\rightarrow Leu}$ and $b_{sol\ Ala-79\rightarrow Val}$ polypeptides (data not shown), were also present in fractions 80−84 (3.3 ± 1.0 units), reflecting binding to the trailing shoulder in the peak in F_1 activity. Importantly, no $b_{sol\ Ala-79\rightarrow Pro}$ was found in fractions eluting from the column prior to the position of uncomplexed polypeptide (Figure 5).

Molecular Mass Determination. Analytical ultracentrifugation using global curve fitting for data from the b_{sol}, $b_{sol\ Ala-79\rightarrow Pro}$, and $b_{sol\ Ala-79\rightarrow Leu}$ polypeptides resulted in a calculated subunit molecular mass of 15.3 kDa, in excellent agreement with that deduced from the sequence of b_{sol} (15.5 kDa). The b_{sol} polypeptide was more than 95% dimeric (with the percentage based on subunit concentration), with a small amount of higher oligomer. The theoretical curve fit to the b_{sol} data in Figure 6 shows the expected result for a mixture that is approximately 96% dimer and 4% tetramer. The distribution for the $b_{sol\ Ala-79\rightarrow Pro}$ polypeptide was monoexponential and consistent with the conclusion that it was monomeric under these experimental conditions (Figure 6). The equilibrium distribution for the $b_{sol\ Ala-79\rightarrow Leu}$ polypeptide was suggestive of a mixture of monomer and oligomer, with the best fit obtained for a composition of 8% dimer (90% confidence interval, 5−11%). Alternatively, a slightly lower percentage of higher oligomer (trimer or tetramer) instead of dimer provides an acceptable fit to the $b_{sol\ Ala-79\rightarrow Leu}$ data. Finally, although analyzed in terms of fixed relative amounts of oligomeric species, the data for the $b_{sol\ Ala-79\rightarrow Leu}$ polypeptide (or b_{sol}) did not rule out an equilibrium association reaction in which the extent of oligomerization was concentration-dependent.

Equalizing Dimer Concentration To Inhibit F_1 Binding to F_0. To demonstrate that dimerization was necessary for binding F_1-ATPase, concentrations of recombinant b_{sol} polypeptides were normalized according to the dimer concentration in reconstitution inhibition assays (see above). At all concentrations of dimer tested, the wild-type b_{sol} and $b_{sol\ Ala-79\rightarrow Leu}$ polypeptides had virtually identical capacities for reducing fluorescence quenching (Figure 7). The $b_{sol\ Ala-79\rightarrow Pro}$ polypeptide had very little effect on fluorescence

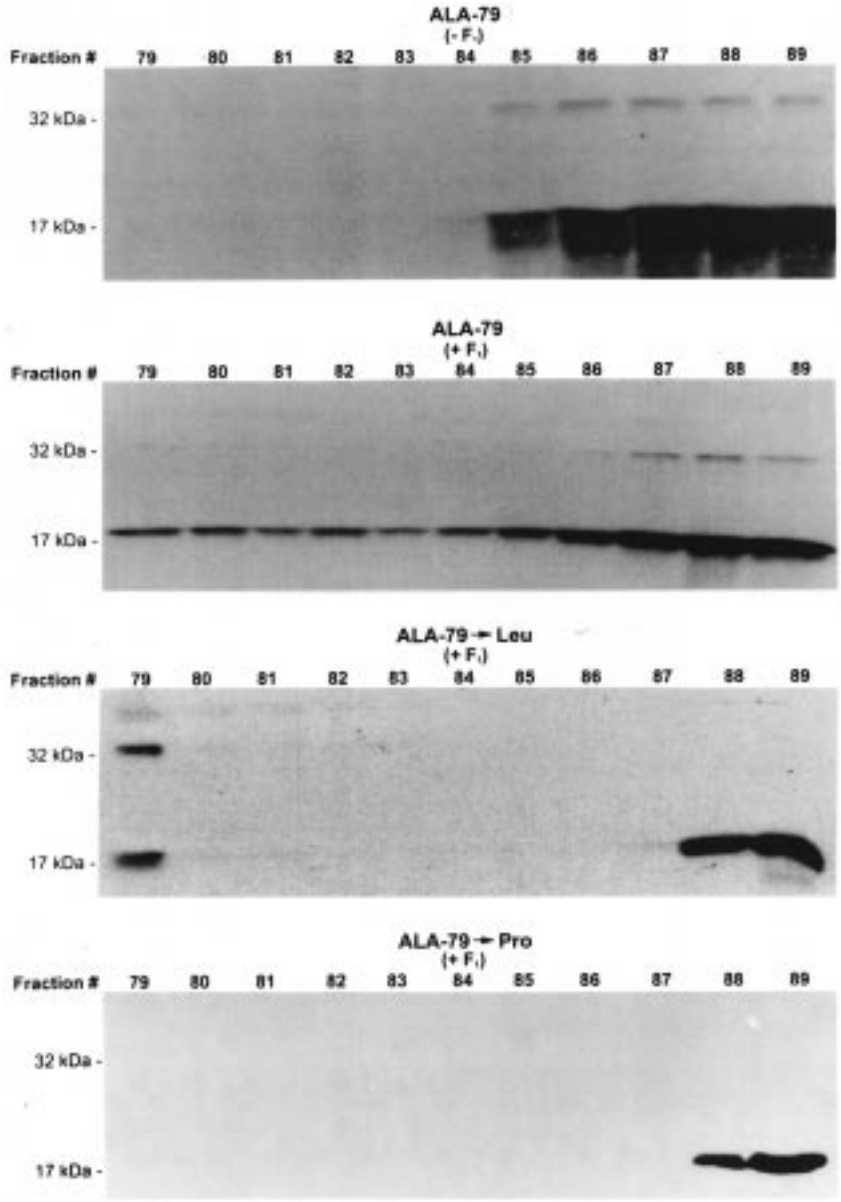

FIGURE 5: Interactions of recombinant b_{sol} polypeptides and F_1-ATPase. F_1-ATPase (480 μg) was mixed with recombinant b_{sol} polypeptides (75 μg) in a volume of 0.2 mL for 30 min at 25 °C. The material was applied to a Superose 12 column equilibrated with MMN buffer (20 mM MOPS-NaOH, 5 mM $MgCl_2$, and 100 mM NaCl at pH 7.4). The column was developed at a flow rate of 0.2 mL/min, and fractions of 0.25 mL were collected. Fractions were reduced in volume using a Jouan RC 10.10 speed vacuum, and an aliquot representing one-third of the fraction was loaded on a 15% SDS−PAGE gel. The protein was transferred to a nitrocellulose membrane and analyzed by immunoblot analysis. The $b_{sol\ Ala-79}$ minus F_1-ATPase experiment was used to indicate where b_{sol} elutes from the column. $b_{sol\ Ala-79}$ and $b_{sol\ Ala-79→Leu}$ bound to F_1-ATPase were also observed in fractions 75−78 (data not shown). In some fractions of wild-type and recombinant polypeptides, a band corresponding to a b_{sol} dimer was observed, despite the use of a denaturing SDS−PAGE gel.

quenching, suggesting that this polypeptide had no significant affinity for binding specifically to F_1-ATPase. The quenching effects are not the result of the protein concentration because bovine serum albumin did not compete with F_1 binding to F_0. These observations strongly argue that formation of a b_{sol} polypeptide dimer was necessary for association with F_1-ATPase.

Nuclear Magnetic Resonance of Model Peptides. NMR was used to examine the secondary structural properties in the vicinity of Ala-79. Model peptides were synthesized for the wild type and the Ala-79 → Pro and Ala-79 → Gln substitutions. The amide proton region of the NOESY spectra for each peptide is shown in Figure 8. Both the wild-type and Ala-79 → Gln peptides contain a series of weak, but easily detectable, sequential (i to $i + 1$) amide NOEs throughout most of the length of each peptide. Although all of the peptides in this work are quite flexible, the pattern of NOEs in the wild type and Ala-79 → Gln are most consistent with a "nascent helix" (*35*). On the other hand, the proline in Ala-79 → Pro appears to prevent the formation of the nascent helix seen in the wild type or Ala-79 → Gln spectra, as shown by the lack of amide to amide NOEs in that peptide (Figure 8). Consideration of both the NMR data and the CD data suggests that the proline mutation disrupts the helix only in a localized region. Moreover, in Ala-79 → Pro, we see no strong evidence of a turn substituting for the nascent helix.

None of the peptides for NMR study showed significant rigidity or strong evidence for turns. The NMR data are most consistent with a helical region around Ala-79 and least

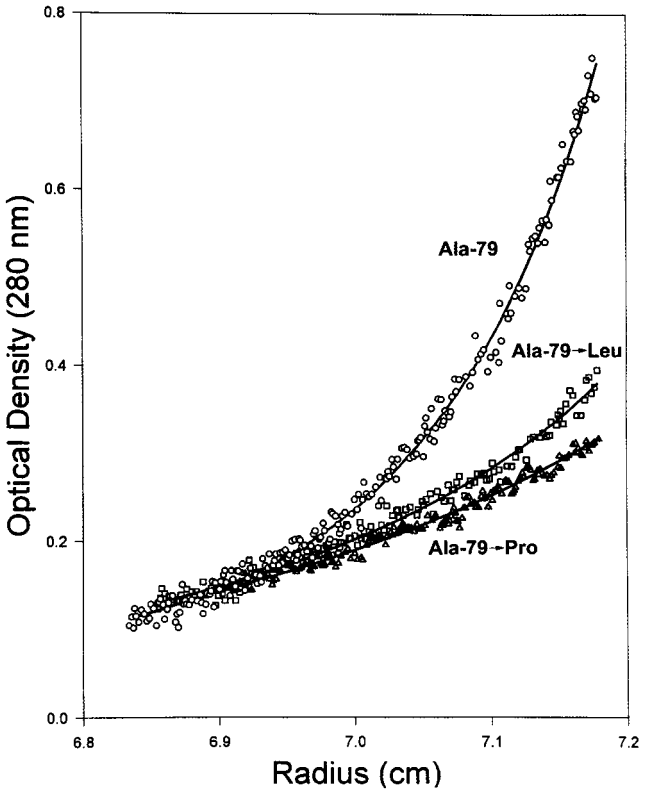

FIGURE 6: Distribution of recombinant b_{sol} polypeptides at sedimentation equilibrium. The concentrations of each protein at equilibrium are shown as a function of radius for b_{sol} (○), $b_{sol\ Ala-79\rightarrow Leu}$ (□), and $b_{sol\ Ala-79\rightarrow Pro}$ (△), with initial concentrations of approximately 0.38, 0.28, and 0.28 mg/mL, respectively. The solid lines are the theoretical curves derived as described in the text and show that each protein is oligomerized to a different extent (i.e. the $b_{sol\ Ala-79\rightarrow Pro}$ polypeptide is monomeric and the wild-type b_{sol} polypeptide is mostly dimeric).

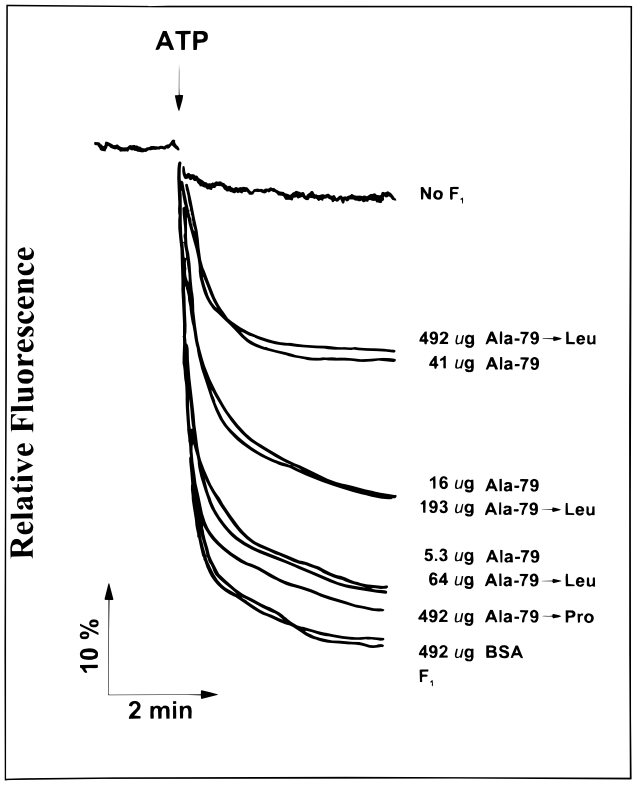

FIGURE 7: Normalization of $b_{sol\ Ala-79}$ and $b_{sol\ Ala-79\rightarrow Leu}$ dimers is required to inhibit reconstitution of F_1 to F_0. F_1-ATPase-depleted membranes (0.125 mg of protein) were reconstituted with 9.8 μg of F_1-ATPase in the presence of $b_{sol\ Ala-79}$ or $b_{sol\ Ala-79\rightarrow Leu}$ polypeptides with similar dimer concentrations as discussed in Figure 4. Membrane energization was determined as described in Experimental Procedures. The concentration of $b_{sol\ Ala-79\rightarrow Leu}$ was approximately 12-fold greater than that of wild-type b_{sol}. The recombinant b_{sol} polypeptides and their concentrations are labeled next to each trace. Bovine serum albumin was used as a control for nonspecific binding.

consistent with a reverse turn. This suggests the *b* subunit may extend up toward the F_1 sector rather than hooking back to the membrane. Although the peptides studied by NMR in this study were quite short and flexible, there are numerous examples of even shorter sequences forming well-defined and fairly rigid turns in aqueous solution (*36*, *37*). On the other hand, we cannot rule out the possibility of a turn or some other type of secondary structure that is induced by interactions within the *b* subunit or the intact enzyme complex.

DISCUSSION

This work describes the effects of amino acid substitutions at Ala-79 on dimerization of polypeptides modeled on the hydrophilic domain of the *b* subunit of F_1F_0 ATP synthase. By circular dichroism, the recombinant polypeptides retained α-helical conformations, suggesting that replacement of Ala-79 with a variety of other amino acids had minimal effects on the overall secondary structure. The NMR results for the wild type and Ala-79 → Gln peptides also provide evidence for helical properties. However, alteration of Ala-79 in b_{sol} resulted in a reduction in formation of the characteristic b_{sol} homodimer. Chemical cross-linking and equilibrium sedimentation experiments provided clear evidence that substitution of proline or polar residues, such as glutamate or glutamine, reduces dimerization below the level of detection. Substitutions of large nonpolar amino acids also resulted in reductions in dimer formation, but to a lesser extent. Apparently, the Ala-79 substitutions alter the distribution between the monomer and dimer. Interestingly, normalization of the concentration of the $b_{sol\ Ala-79\rightarrow Leu}$ dimers to the concentration on wild-type b_{sol} dimers resulted in virtually identical binding to F_1-ATPase.

Earlier biochemical characterization studies of *uncF(b)* gene mutations affecting Ala-79 suggested a defect in F_1F_0 ATP synthase assembly (*15*, *38*). Expression of the mutant genes from the chromosome resulted in reduced steady-state levels of F_1F_0 ATP synthase; overexpression of the mutant genes using an inducible plasmid resulted in restoration of abundant levels of enzyme in an *uncF(b)* gene deletion strain. The recombinant proteins were capable of being productively incorporated into the enzyme complex, but the assembly of the F_1F_0 ATP synthase was efficient only in the presence of excess levels of the altered *b* subunits. The more deleterious mutations in vivo correspond to the substitutions in b_{sol} polypeptides which failed in the dimerization assays, and the intermediate phenotypes in vivo are those that have detectable levels of dimerization in the model system. Therefore, it seems clear that the primary effect of the replacement of Ala-79 is on formation of the *b* subunit dimer. A similar conclusion was drawn for the Ala-128 → Asp substitution studied by Howitt et al. (*16*).

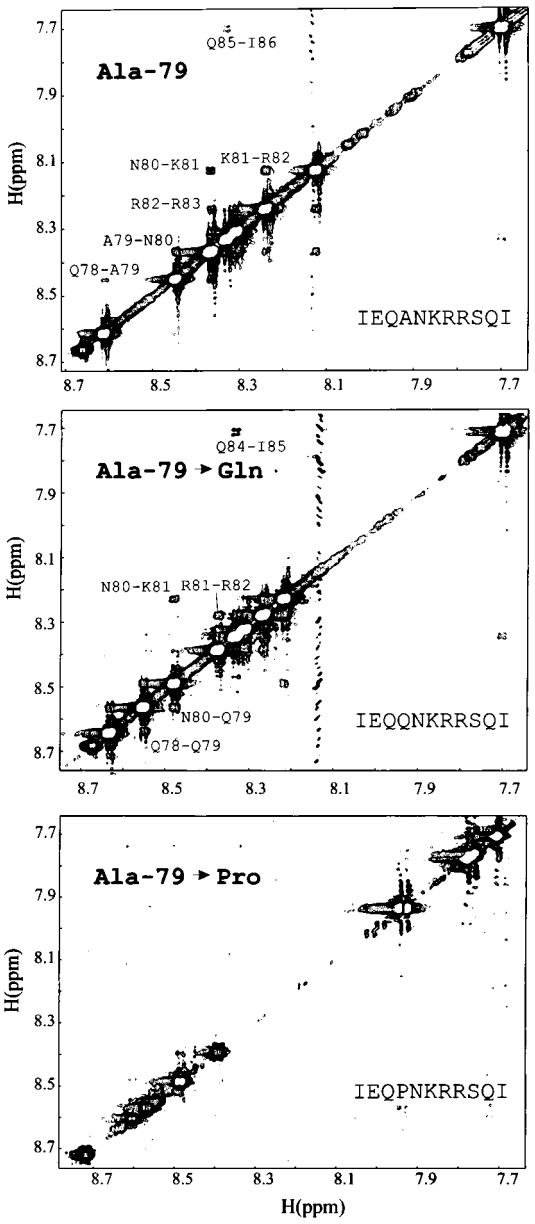

FIGURE 8: Amide region of NOESY spectra for Ala-79, Ala-79 → Gln, and Ala-79 → Pro model peptides. All experiments were performed at 4 °C. Data were collected with NOE mixing times of 200 ms (wild type), 150 ms (Ala-79 → Gln), and 250 ms (Ala-79 → Pro). Mixing times as short as 150 ms (data not shown) were used on the wild-type sample to rule out spin diffusion effects. The peptide sequences are indicated on each panel.

Beyond the fact that dimerization is affected by single amino acid substitutions, we have shown that dimerization of $b_{\text{sol Ala−79→Leu}}$ appears to be directly proportional to its capacity for binding F_1. This provides an important insight into the nature of the defects resulting from mutations affecting $b_{\text{Ala−79}}$. Ala-79 is probably not at the interface between the *b* subunit and F_1. If the amino acid substitutions altered the interface, then one would have expected that the native b_{sol} dimer would have displayed a higher affinity for F_1-ATPase than the $b_{\text{sol Ala−79→Leu}}$ dimer. Although a quantitative determination of binding affinity was not performed, the assay systems used here were sufficient to detect marked changes in affinity for F_1-ATPase. The observation that the secondary structure was not altered sufficiently to be reflected in the circular dichroism spectra from even the most nonconservative of substitutions, like $b_{\text{sol Ala−79→Lys}}$, suggests that the structural defects are localized within the protein. Together, the evidence suggests that substitution of Ala-79 results in a short-range effect in an important structural determinant of a domain necessary for the dimerization of the two *b* subunits in F_1F_0 ATP synthase, but has no apparent influence on the areas of the *b* subunit involved in interaction with F_1.

Perhaps a more profound implication of the proportionality of dimer formation to F_1-ATPase binding is that this observation suggests that *b* subunit monomers may have very little affinity for F_1. The logical extension of this idea is that formation of the *b* subunit dimer is a necessary event preceding F_1 binding to F_0 in the assembly of the enzyme complex. This is not consistent with the pathway for concerted assembly of F_1F_0 ATP synthase proposed in an older model (*39*). Apparently, F_1F_0 ATP synthase assembly requires stable association of F_1 with an intact *b* subunit dimer in F_0. The *b* subunit dimer is likely to be resident in an intact F_0 sector prior to F_1 binding. Contact between the *b* subunit and the F_1 δ subunit then has some, as yet undefined, effect on F_0, resulting in activation of the proton channel (*40*, *41*). A major remaining weakness in our understanding of F_1F_0 ATP synthase is the order of events in assembly in vivo and the nature of the subunit interactions involved in this process.

REFERENCES

1. Nakamoto, R. K. (1996) *J. Membr. Biol. 151*, 101−111.
2. Deckers-Hebestreit, G., and Altendorf, K. (1996) *Annu. Rev. Microbiol. 50*, 791−824.
3. Weber, J., and Senior, A. E. (1997) *Biochim. Biophys. Acta 1319*, 19−58.
4. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature 370*, 621−628.
5. Duncan, T. M., Bulygin, V. V., Zhou, Y., Hutcheon, M. L., and Cross, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10964−10968.
6. Sabbert, D., Engelbrecht, S., and Junge, W. (1996) *Nature 381*, 623−625.
7. Sabbert, D., Engelbrecht, S., and Junge, W. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 4401−4405.
8. Noji, H., Yasuda, R., Yoshida, M., and Kinosita Jr., K. (1997) *Nature 386*, 299−302.
9. Senior, A. E. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 7−41.
10. Dunn, S. D. (1992) *J. Biol. Chem. 267*, 7630−7636.
11. Wilkens, S., Dunn, S. D., and Capaldi, R. A. (1994) *FEBS Lett. 354*, 37−40.
12. Jans, D. A., Fimmel, A. L., Langman, L., James, L. B., Downie, J. A., Senior, A. E., Gibson, F., and Cox, G. B. (1983) *Biochem. J. 211*, 717−726.
13. Jans, D. A., Fimmel, A. L., Hatch, L., Gibson, F., and Cox, G. B. (1984) *Biochem. J. 221*, 43−51.
14. Porter, A. C. G., Kumamoto, C., Aldape, K., and Simoni, R. D. (1985) *J. Biol. Chem. 260*, 8182−8187.
15. McCormick, K. A., Deckers-Hebestreit, G., Altendorf, K., and Cain, B. D. (1993) *J. Biol. Chem. 268*, 24683−24691.
16. Howitt, S. M., Rodgers, A. J., Jerrery, P. D., and Cox, G. B. (1996) *J. Biol. Chem. 271*, 7038−7042.
17. Deckers-Hebestreit, G., and Altendorf, K. (1986) *Eur. J. Biochem. 161*, 225−231.
18. Deckers-Hebestreit, G., Simoni, R. D., and Altendorf, K. (1992) *J. Biol. Chem. 267*, 12364−12369.
19. Markwell, M. A. K., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206−210.
20. Weber, J., Wilke-Mounts, S., and Senior, A. E. (1994) *J. Biol. Chem. 269*, 20462−20467.

21. Aris, J. P., Klinosky, D. J., and Simoni (1985) *J. Biol. Chem. 260*, 11207−11215.
22. Tamarappoo, B. K., Handlogten, M. E., Lane, R. O., Serrano, M. A., Dugan, J., and Kilberg, M. S. (1992) *J. Biol. Chem. 267*, 2370−2374.
23. Lee, S. P., Xiao, j., Knutson, J. R., Lewis, M. S., and Han, M. K. (1997) *Biochemistry 36*, 173−180.
24. Knott, G. D. (1979) *Comput. Programs Biomed. 10*, 271−280.
25. Bubb, M. R., Lewis, M. S., and Korn, E. D. (1991) *J. Biol. Chem. 266*, 3820−3826.
26. Cain, B. D., and Simoni, R. D. (1986) *J. Biol. Chem. 261*, 10043−10050.
27. Callihan, D., West, J., Kumar, S., Schweitzer, B. I., and Logan, T. M. (1996) *J. Magn. Reson., Ser. B 112*, 82−85.
28. Kumar, A., Ernst, R. R., and Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1.
29. Braunschweiler, L., and Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521.
30. Marion, D., Ikura, M., and Bax, A. (1989) *J. Magn. Reson. 84*, 425.
31. Delaglio, F., Grzesiek, S., Vuister, G., Zhu, G., Pfeifer, J., and Bax, A. (1995) *J. Biomol. NMR 6*, 277−293.
32. Wüthrich, K. (1996) *NMR of Proteins and Nucleic Acids*, John Wiley and Sons, New York.
33. Johnson, B. A., and Blevins, R. A. (1994) *J. Biomol. NMR 4*, 603−614.
34. Hoppe, J., Friedl, P., Schairer, H. U., Sebald, W., von Meyenburg, K., and Jorgensen, B. B. (1983) *EMBO J. 2*, 105−110.
35. Dyson, H. J., Rance, M., Houghten, R. A., Wright, P. E., and Lerner, R. A. (1988) *J. Mol. Biol. 201*, 201−217.
36. Yao, J., Dyson, J., and Wright, P. E. (1994) *J. Mol. Biol. 243*, 754−766.
37. Sieber, V., and Moe, G. R. (1996) *Biochemistry 35*, 181−188.
38. McCormick, K. A., and Cain, B. D. (1991) *J. Bacteriol. 173*, 7240−7248.
39. Cox, G. B., Jans, D. A., Fimmel, A. L., Gibson, F., and Hatch, L. (1984) *Biochim. Biophys. Acta 768*, 201−208.
40. Angov, E., Ng, T. C. N., and Brusilow, W. S. (1991) *J. Bacteriol. 173*, 407−411.
41. Monticello, R. A., Angov, E., and Brusilow, W. S. (1992) *J. Bacteriol. 174*, 3370−3376.

BI972309+