# Proton-Translocating Carboxyl of Subunit *c* of $F_1F_o$ $H^+$-ATP Synthase: The Unique Environment Suggested by the p$K_a$ Determined by $^1H$ NMR†

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ABSTRACT: Subunit *c* of the $H^+$-transporting $F_1F_o$ ATP synthase (EC 3.6.1.34) is thought to fold across the membrane as a hairpin of two α helices with a conserved Asp/Glu residue, centered in the second membrane-spanning helix, which is thought to function in $H^+$ translocation. NMR studies indicate that the purified subunit *c* from *Escherichia coli* is also folded as a hairpin in a chloroform/methanol/$H_2O$ (4:4:1) solvent mixture [Girvin, M. E., & Fillingame, R. H. (1993) *Biochemistry 32*, 12167−12177] and that the conserved Asp remains uniquely reactive in this solvent mixture [Girvin, M. E., & Fillingame, R. H. (1994) *Biochemistry 33*, 665−674]. The p$K_a$ of Asp61 is of interest because of its unique reactivity and because it is thought to protonate and deprotonate during each proton translocation cycle. We have determined the p$K_a$ value of the carboxyl group of the functional Asp in wild type and two functional, mutant subunit *c* proteins, i.e. the Ala24 → Asp (D24D61) and the Ala24 → Asp/Asp61 → Asn (D24N61) mutant proteins. The p$K_a$ values were determined by $^1H$ NMR spectroscopy by measuring changes in the α and β proton chemical shifts by constant time two-dimensional (2D) correlated spectroscopy. The p$K_a$ of Asp61 in the purified wild type protein was 7.1. This p$K_a$ was significantly higher than the p$K_a$ of the other two Asp residues, i.e. Asp7 and Asp44 which were 5.4 and 5.6, respectively. The p$K_a$ of the two Glu residues in the protein were determined by 2D total correlation spectroscopy and found to be approximately 5.5. In the D24D61 mutant protein, the p$K_a$ values of both Asp24 and Asp61 were abnormally high, i.e. 7.1 and 6.9, respectively. In the D24N61 mutant protein, where Asp24 functions in proton translocation, the p$K_a$ of the functional Asp24 was 6.9. These observations suggest that the protein folds such that the local environment around Asp61 (or Asp24) is more hydrophobic than in the general environment of the organic solvent. The changes in chemical shift of sequentially assigned protons of other residues were followed as a function of pH as a general measure of structural changes in the protein. The analysis indicates a global conformational change in the protein coincident with the deprotonation of Asp61. The most pronounced effect of deprotonation is seen in the polar loop region and in the N-terminus. The conformational changes in this region of the protein, which are coupled to protonation/deprotonation of Asp61, may mimic those taking place during ATPase-coupled proton translocation by the $F_1F_o$ complex.

The proton-translocating $F_1F_o$ ATP synthase of *Escherichia coli* consists of an intrinsic membrane sector, $F_o$, which forms a proton channel across the cytoplasmic membrane and an extrinsic sector, $F_1$, which catalyzes ATP synthesis or hydrolysis (Fillingame, 1990). The subunit structure is complex, that is, subunit compositions of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ for $F_1$ and $a_1b_2c_{10}$ for $F_o$ (Foster & Fillingame, 1982). The subunits *a* and *c* in the $F_o$ sector appear to be essential for proton translocation. Subunit *c* is a protein of 79 amino acid residues containing two hydrophobic segments of more than 20 amino acids at the N- and C-terminal ends that are thought to span the lipid bilayer. Hoppe and Sebald (1984) proposed that the protein folded in the membrane like a hairpin, where the membrane-spanning regions were assumed to be α helices. These hydrophobic segments are separated by a more polar loop region of about 18 amino acids that is thought to be important for the coupling of $F_o$ to $F_1$ (Mosher et al., 1985; Fraga et al., 1994; Zhang et al., 1994).

Asp61 of *E. coli* subunit *c* lies in the middle of the transmembrane helix-2. This residue is thought to be a critical component of the proton conduction system of $F_o$. It is proposed that the essential carboxyl group protonates and deprotonates during each proton translocation cycle with an alteration between a high- and a low-p$K_a$ form (Fillingame, 1990). Several lines of evidence support this contention. Mutation of Asp61 to Gly or Asn results in a loss of function (Hoppe et al., 1980a, 1982; Mosher et al., 1983). The fact that the Asn substitution abolishes function suggests a requirement for an ionizable group at this position. A Glu substitution here leads to low activity and suggests that the

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[1] Abbreviations: 2D, two-dimensional; C/M/W solvent, $C^2HCl_3$/$C^2H_3O^2H$/$^2H_2O$ (4:4:1) mixture made 50 mM in NaCl; COSY, 2D correlated spectroscopy; CTCOSY, 2D constant time correlated spectroscopy; DCCD, *N,N'*-dicyclohexylcarbodiimide; NCCD, *N*-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N'*-cyclohexylcarbodiimide; pH*, the glass electrode pH reading without correction for the deuterium isotope effect; SDS, sodium dodecyl sulfate; TOCSY, 2D total correlation spectroscopy.

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microenvironment and/or the p$K_a$ of the carboxyl group may be essential for function (Miller et al., 1990). Asp61 is also subject to specific modification by DCCD.[1] This modification blocks proton translocation and provided the earliest evidence that the carboxyl group may be required in the protonation/deprotonation step (Sebald & Hoppe, 1981; Fillingame, 1990).

Two lines of evidence suggest that residues 24 and 28 in the first helix may lie close to the DCCD reactive Asp61 residue in the second helix as the protein folds in the membrane. First, the rate of reaction of DCCD with Asp61 is slowed in A24S and I28T mutants (Fillingame et al., 1991; Hoppe et al., 1980b). Second, the essential carboxyl group can be shifted from position 61 of the second helix to position 24 of the first helix with retention of function (Miller et al., 1990). This was initially shown with the Ala24 → Asp/Asp61 → Gly (D24G61)[2] double mutant. Subsequently, two other double mutants, D24N61 and D24D61, were also shown to be active (Zhang & Fillingame, 1994). The function of the D24D61 mutant is pH dependent; activity appears to depend upon titration of a group with a predicted p$K_a$ of about 7.4.

NMR experiments with isolated subunit *c* indicate that its structure in a $C^2HCl_3/C^2H_3O^2H/^2H_2O$ (4:4:1) solvent mixture made 50 mM in NaCl (C/M/W solvent) is similar to that in the membrane (Girvin & Fillingame, 1993, 1994). A hairpin structure for subunit *c* was suggested, on the basis of NOEs between residues at both ends of the two proposed membrane-spanning α helices. In addition, Asp61 is specifically modified with DCCD in C/M/W solvent, and the rate of the modification is decreased in the I28T mutant protein, as it is in the membrane. These results suggest that the folding and key chemical properties of subunit *c* may be preserved in C/M/W solvent.

We determined the p$K_a$ of the functioning carboxyl group in the wild type protein and the functional D24D61 and D24N61 mutant proteins. These mutant proteins were examined to determine if there were relationships between p$K_a$, position, and function. Our results indicate that the p$K_a$ of Asp61 in the wild type protein is significantly higher than the p$K_a$ of the other two Asp residues in the protein (i.e. Asp7 and Asp44). Previous structural studies suggest that the Asp61 carboxyl may lie within a pocket of hydrophobic residues (Girvin & Fillingame, 1994, 1995), and this may account for the unusually high p$K_a$ value. In the D24D61 mutant protein, Asp24 also has a high p$K_a$, i.e. 7.1; a high p$K_a$ was also predicted for Asp24 from studies of the D24D61 mutant (Zhang & Fillingame, 1994). In the D24N61 mutant protein, the p$K_a$ of Asp24 was 6.9. The essential carboxyl therefore has a high p$K_a$ regardless of its placement at position 61 or position 24. Finally, deprotonation of the essential Asp appears to result in global conformational changes in the loop region of the protein which may be of functional significance.

## EXPERIMENTAL PROCEDURES

*E. coli Strains and Growth Media.* Strain DF540 is a derivative of strain JM83 (Messing & Vieira, 1982) which carries the wild type chromosomal *unc* operon (ATP−synthase complex) and a pUC18-derived plasmid encoding the subunit *c* gene (Fraga & Fillingame, 1991). Strain JM83 was transformed with plasmid pDF255 [a pUC18 vector carrying a *Bam*H1 (1727)-*Hpa*I (2376) *unc* DNA fragment encoding the complete *uncE* (subunit *c)* gene] to generate strain DF540. Strain DF540 was grown in M63 minimal medium (Miller, 1972) (pH 7.0) containing 10% Luria-Bertani medium (i.e. 0.1% tryptone, 0.1% NaCl, and 0.05% yeast extract), 1% glycerol (w/v), 1 mM L-proline, 2 mg/L thiamine, and 100 mg/L ampicillin. This strain overproduces subunit *c* protein by approximately 12−15-fold when cells are harvested at the beginning of the stationary phase. Wild type subunit *c* protein was isolated from these cells. The yield was 1.5 mg of Lowry-determined protein (0.375 μmol) per gram of wet cell paste.

The mutant strains in this study are FA274 (YZ274/pFA111) and FA275 (YZ275/pMEG130). Strains YZ274 (D24D61) and YZ275 (D24N61) are the chromosomal mutant strains as described in Zhang and Fillingame (1994). The overproducing plasmids, pFA111 (D24D61) and pMEG130 (D24N61), were constructed as described in Fillingame et al. (1991). These plasmids contain the D24D61 or D24N61 *uncE* genes in the *Bam*H1 (1727)-*Hpa*I (2162) DNA fragment in a pCP35-derived vector. Strains FA274 (D24D61) and FA275 (D24N61) were grown in M63 minimal medium (pH 7.0) containing 10% Luria-Bertani medium, 1% D-glucose, 4 mg/L thiamine, 0.2 mM arginine, 0.2 mM uracil, 40 μM 2,3-dihydroxybenzoic acid, and 100 mg/L ampicillin.

*Subunit c Isolation from Whole Cells.* Subunit *c* protein was purified from whole cells by extraction into chloroform/methanol (2:1) followed by precipitation with diethyl ether (Fillingame, 1976). Further purification by CM-cellulose column chromatography was carried out as described (Graf & Sebald, 1978; Hermolin & Fillingame, 1989), where the protein was eluted with chloroform/methanol/$H_2O$ (4:4:1). The eluted protein in chloroform/methanol/$H_2O$ (4:4:1) was then loaded onto and eluted from an LH-20 Sephadex gel column to transfer the protein into chloroform/methanol (2:1) prior to NMR sample preparation. In the case of the D24D61 mutant protein, a DEAE anion exchange column was used in lieu of CM-cellulose chromatography, and the protein was eluted by an ammonium acetate salt gradient as described (Fillingame, 1976). This was necessary because of indications of minor heterogeneities in the initial mutant protein prepared by CM-cellulose chromatography. The protein concentration was measured by a modified form of the Lowry method with SDS in the assay (Fillingame, 1976; Hermolin & Fillingame, 1989), and the molarity of subunit *c* was calculated as described (Girvin & Fillingame, 1993). The protein purity was examined on an AX300 anion exchange HPLC column (Synchropak, Linden, IN) using an ammonium acetate gradient in chloroform/methanol/$H_2O$ (4:4:1) (Hermolin & Fillingame, 1989).

*NMR Sample Preparation.* Subunit *c* was transferred from chloroform/methanol (2:1) to the deuterated solvent [$C^2HCl_3/C^2H_3O^2H/^2H_2O$ (4:4:1) made 50 mM in NaCl (C/M/W solvent)] as described by Girvin and Fillingame (1993), where the final concentration of protein was 1.5−2 mM. Tetramethylsilane was added as internal reference.

*Activity Measurement by Glass Electrode.* The following experiment was done to show that the $H^+$ activity reading

[2] Henceforth, mutant and wild type proteins will be identified by the amino acid residues at positions 24 and 61. By this nomenclature, wild type is A24D61, Ala24 → Asp is D24D61, and Ala24 → Asp/Asp61 → Asn is D24N61.

given by a combination pH glass electrode was insensitive to the solvents used here. A two-phase solvent system was generated by mixing chloroform/methanol/$H_2O$ in proportions of 8:4:3 and the pH of the two phases measured at equilibrium. In several trials with different electrodes, the pH of the upper phase with a chloroform/methanol/$H_2O$ composition of 3:47:48 did not differ from the pH of the lower phase with a chloroform/methanol/$H_2O$ composition of 86:14:3 by more than 0.05 pH unit.

The pH* of the NMR sample was adjusted by the addition of 0.1−1 N $NaO^2H$ or $^2HCl$ (Aldrich Chemical Co.). pH* denotes a glass electrode reading without correction for the deuterium isotope effect. The pH* of the sample was adjusted prior to NMR experiments and was remeasured after the experiments to ensure that it remained constant during the experimental procedure. Generally, the two pH* readings agreed within <0.1 pH* unit and did not vary in a systematic fashion from sample to sample. In the figures shown, the final pH value is the one plotted. The experiments were done in the pH* range of 3.3 to 8.0. The protein precipitated at pH* greater than 8. To check the reversibility of the pH* titration, experiments were conducted where the pH* was taken to pH 7.8 or 4.8 and brought back slowly to the starting pH* (5.4−5.8). The chemical shifts of all cross-peaks were found to be the same as in the original sample where the pH was not perturbed.

*NMR Experiments and Data Processing.* All $^1H$ NMR spectra were recorded at 298 K on a Bruker AM-500 or AM-600 spectrometer. All chemical shifts were measured relative to tetramethylsilane. The sweep widths were 5559 Hz (on the AM-500) and 6024 Hz (on the AM-600) for all experiments. The TOCSY experiment was done as described by Shaka (1988) and Bax (1989) using the DIPSI-2 pulse train and two 1.5 ms trim pulses. A mixing time of 65 ms was empirically determined to give the optimal magnetization transfer from α to γ protons of L-glutamic acid. The constant time COSY (CTCOSY) experiment employed a $90x - (tc + t1)/2 - 180x - (tc - t1)/2 - 90x$ acquisition sequence (Girvin, 1994), where tc is constant time. Constant time for all experiments was set to 38 ms. Each FID was composed of 2048 data points and was the sum of 16−32 scans for CTCOSY. A total of 380−400 FIDs were collected. Two dummy scans were used for each FID. The relaxation delay was set to 2 s. All data were processed using Felix 2.05 software (Hare Research, Bothell, WA) on a Silicon Graphics IRIS computer. All spectra were processed as 2048 × 1024 (or 2048) data points along t2 and t1 axes, respectively, and were multiplied by a shifted sine bell in both dimensions prior to Fourier transformation. Linear prediction was used to extend the data to 650 t1 points. The rolling base line was corrected by the frequency−domain convolution difference according to Fortier et al. (1991). Complete removal of diagonal peaks was accomplished via a digital filtering scheme in the t2 dimension (Friedrichs et al., 1991).

*$pK_a$ Determination and Error Analysis.* The $pK_a$ determination was performed using a nonlinear least squares fit of the experimental data to the equation

$$pH^* = pK_a + \log[(\delta_a - \delta)/(\delta - \delta_b)] \qquad (1)$$

where $\delta$ is the chemical shift of the proton at varying pH* and $\delta_a$ and $\delta_b$ are chemical shifts of the proton with the titrating group in fully acidic or basic form, respectively (Bradbury & Brown, 1973; Brown & Bradbury, 1975). Three parameters, the chemical shift values at high and low pH plateaus and the $pK_a$ value, were optimized in fitting the experimental data to eq 1. The error analysis was calculated by using the nonlinear multivariate scant or false position (DUD) method (Ralston & Jennrich, 1978) in SAS (statistical analysis software) version 6.03. A t-distribution with 17 degrees of freedom was used. The 95% confidence limits, rounded to the nearest 0.1 pH unit, were chosen to indicate the limits of error. Systematic variation was not apparent from the residual plots. The data for the three sets of aspartyl $H^\beta$ and the C-terminal Ala $H^\alpha$ in the wild type protein were also analyzed by the Hill equation and showed Hill constants of 0.9, 0.9, 0.9, and 1.1, respectively. The lack of cooperativity indicated justifies the use of eq 1, where the Hill constant is defined as 1.

*Control Peptide.* The peptide Gly-Gly-Asp-Ala (purchased from Bachem AG, Liestal, Switzerland) was used as a model compound for comparison of the $pK_a$ of Asp and C-terminal carboxyl groups in the C/M/W solvent mixture with that in aqueous 50 mM NaCl. The NMR samples were 10 mM peptide in $^2H_2O$ containing 50 mM NaCl or 2 mM peptide in C/M/W solvent. Tetramethylsilane was added and used as an internal reference. A series of one-dimensional (1D) proton NMR experiments were collected at different pH* values. In addition, a 2D CTCOSY experiment was performed to verify the connectivities.

## RESULTS

*$pK_a$ of Asp Carboxyls in Wild Type (A24D61) Subunit c.* The $^1H$ spin systems for the three Asp residues in the subunit *c* have been identified (Girvin & Fillingame, 1993). We determined the $pK_a$ of the Asp side chain carboxyl groups by following changes in chemical shift of the $H^\alpha$ and $H^\beta$ protons as a function of solution pH*. The expected change in the chemical shift for the β proton of an aspartyl residue undergoing deprotonation is 0.2−0.27 ppm in the upfield direction in aqueous solution (Bundi & Wuthrich, 1979), whereas the expected change in α proton chemical shift varies from 0.06 to 0.2 ppm in the upfield direction (Bartik et al., 1994). The change in chemical shift for the α proton is generally smaller than the change in chemical shifts of the β protons due to the greater inductive effect of the carboxyl group on the neighboring β protons.

Figure 1 shows contour plots of the Asp/Asn $H^\alpha$−$H^\beta$ region of CTCOSY spectra at 600 MHz for wild type subunit *c* protein. The pH* titration experiments were carried out between pH* 3.3 and 8.0. The pH* values shown demonstrate the range of changes in chemical shifts for different carboxyl groups. In the plots shown, the chemical shift of the β proton of the Asp61 residue changed by 0.2 ppm in the upfield direction as the pH was increased from 5.8 to 7.6. In comparison, the $H^\beta$ chemical shifts of both Asp7 and Asp44 change significantly at a lower pH* range (compare pH* 4.1 to 6.9). The $pK_a$ values were determined by a nonlinear regression fit of the data to eq 1. The complete series of titration points for the Asp residues are shown in Figure 2. The $pK_a$ values determined by this process were 5.4 and 5.6 for Asp7 and Asp44, respectively, and 7.1 for Asp61. The results of this and other experiments are summarized in Table 1.

The $pK_a$ values from changes in the chemical shift of the α proton were also determined (Figure 2). The $pK_a$ values

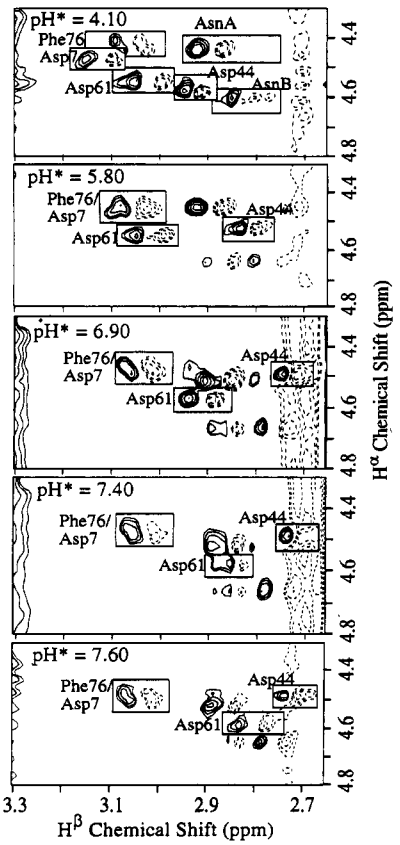


FIGURE 1: CTCOSY spectra demonstrating the range in changes in chemical shift for different Asp groups as the pH* is varied. Contour plots of the Asp H$^\alpha$–H$^\beta$ region of 600 MHz $^1$H CTCOSY of wild type subunit *c* in C/M/W solvent mixture at varying pH* are shown. The H$^\beta$ cross-peaks of Asp7 and Asp44 change between pH* 4.1 and 6.9, whereas the Asp61 cross-peak changes between pH* 5.8 and 7.6. The solid and dashed contours indicate the positive and negative levels, respectively, for the antiphase cross-peaks in the t2 dimension.

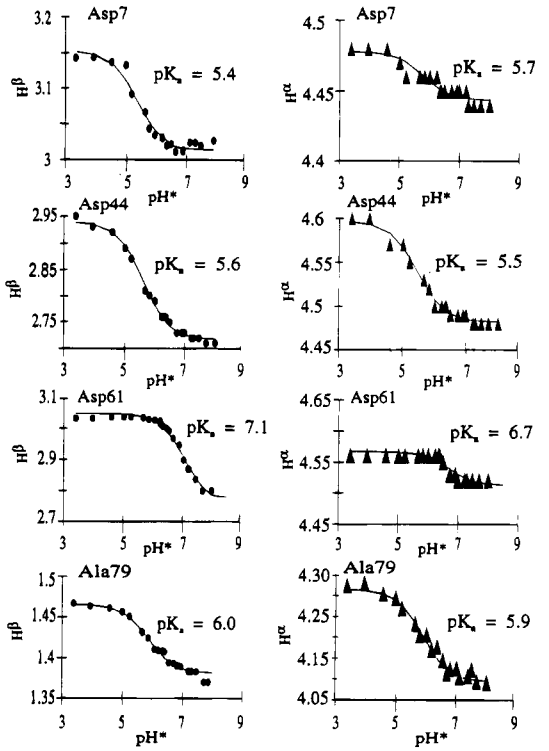


FIGURE 2: Asp61 has an unusually high p$K_a$ compared to other Asp carboxyls of subunit *c*. pH* titration of H$^\beta$ cross-peaks of Asp7, Asp44, Asp61, and the C-terminal Ala79 α-carboxyl for wild type subunit *c* in C/M/W solvent mixture. The solid line indicates the theoretical curve fit to the experimental points using the nonlinear fitting procedure described under Experimental Procedures. The solid circles and triangles indicate experimental data for H$^\beta$ and H$^\alpha$ chemical shifts.

for Asp7, Asp44, and Asp61 were determined to be 5.7, 5.5, and 6.7, respectively. The changes in the H$^\alpha$ chemical shift for Asp7 and Asp61 were very small, i.e. <0.05 ppm, versus the >0.1 ppm for the change in chemical shift of the α proton of Asp44. The small change in the H$^\alpha$ chemical shift may indicate an unusually confining conformation. Because the change in chemical shift is small, it likely accounts for the greater deviation between these p$K_a$ values and those determined from the β protons. The p$K_a$ determined from the β protons would appear to be more reliable since the change in chemical shift is larger.

*p$K_a$ of Other Carboxyls in Wild Type (A24D61) Subunit c.* Glu, Gln, and a few of the Met residues are not specifically assigned from 2D $^1$H NMR (Girvin & Fillingame, 1993). The region of the COSY spectrum showing the α to β and β to γ proton connectivities is crowded due to the presence of eight Met groups. We used a TOCSY experiment to look at α to β and α to γ proton connectivities (α to γ connectivities are normally in a less overlapped area). Upon variation of the pH*, two sets of cross-peaks showed a consistent change of about 0.1 ppm in α to β and 0.2 ppm in α to γ proton chemical shifts (Figure 3). There are only two Glu residues in subunit *c*, Glu2 and Glu37, which are designated as GluA and GluB in this study. The p$K_a$ values for CHγ of GluA and GluB shown here were calculated from data on the wild type protein, and additional data on a D61E mutant protein, since the chemical shifts appear to change identically in the two proteins. The p$K_a$ values were both determined to be 5.5 (Figure 4).

Table 1: Summary of p$K_a$* Values of Carboxyl Groups in the Subunit[c]

| residue/ resonance | protein preparation: wild type 1 | wild type 2 | D24D61 1 | D24D61 2 | D24N61 |
|---|---|---|---|---|---|
| Asp7 Hβ | 5.4 ± 0.4[a] | 5.8 ± 0.2 | 5.4 ± 0.5 | 5.8 ± 0.4 | 6.4 ± 0.2 |
| Asp24 Hβ | – | – | 7.1 ± 0.1 | 7.1 ± 0.1 | 6.9 ± 0.2 |
| Asp44 Hβ | 5.6 ± 0.1 | 5.6 ± 0.1 | 5.6 ± 0.1 | 5.5 ± 0.1 | 5.5 ± 0.2 |
| Asp61 Hβ | 7.1 ± 0.2 | 7.0 ± 0.2 | 6.9 ± 0.3 | 6.8 ± 0.2 | – |
| Ala79 Hα | 5.9 ± 0.2 | 5.7 ± 0.2 | 5.8 ± 0.1 | 5.8 ± 0.1 | 5.9 ± 0.1 |
| Ala79 Hβ | 6.0 ± 0.2 | 5.9 ± 0.2 | 5.7 ± 0.1 | 5.8 ± 0.1 | 5.9 ± 0.1 |
| GluA Hγ | – | 5.5 ± 0.3 | – | – | – |
| GluB Hγ | – | 5.5 ± 0.3 | – | – | – |

[a] p$K_a$ was calculated by a nonlinear DUD analysis in SAS using a t-distribution with 17 degrees of freedom. The error limits indicate a range of 95% confidence.

*p$K_a$ of C-Terminal Ala79 Carboxyl in Wild Type (A24D61) Subunit c.* Contour plots of the alanine region of the CTCOSY spectra were compared at varying pH* (see supporting information for example plots). Five Ala groups are not specifically assigned by 2D $^1$H NMR (Girvin & Fillingame, 1993). These are Ala20, Ala21, Ala39, Ala40, and Ala79. The pH dependent changes in chemical shifts of H$^\alpha$ and H$^\beta$ of AlaA suggest that it is the C-terminal residue with a titratable carboxyl group. The chemical shift of the α and β protons of Ala79 (AlaA) start to change at pH* 4.5. The α proton cross-peak changes by >0.2 ppm, and

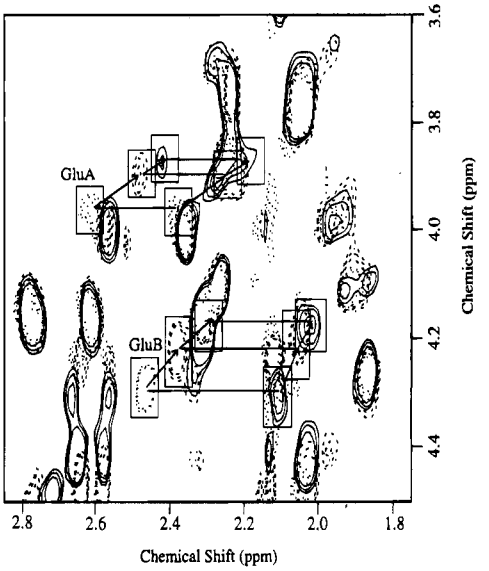


FIGURE 3: Apparent titration of the two glutamyl $\gamma$-carboxyl groups in wild type subunit *c*. Contour plots of the Met/Glx region in a $^1$H TOCSY experiment at 600 MHz at three different pH* values: pH* 4.7, dotted lines; pH* 5.8, dashed lines; and pH* 7.5, solid lines. $\alpha$ to $\beta$ and $\alpha$ to $\gamma$ protons are boxed and connected by solid lines at each pH* point. Titrating residues are designated as GluA and GluB.

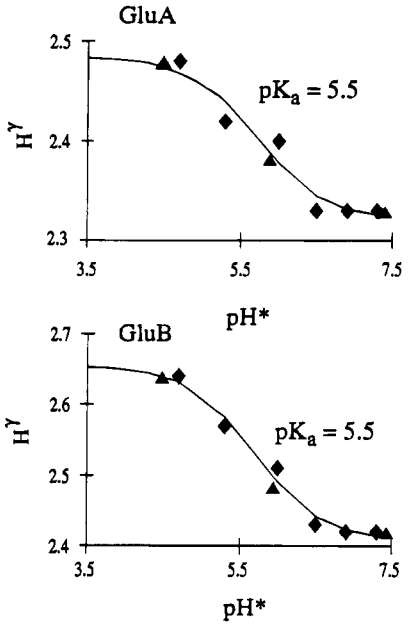


FIGURE 4: pH* titration of $H^\gamma$ chemical shift for Glu residues in the wild type protein. The solid line shows the best fit of the data to eq 1. The solid triangles are experimental values obtained with the wild type protein (Figure 3), and the solid diamonds are data from the Asp61 → Glu mutant protein.

its $\beta$ proton shifts by about 0.1 ppm. The p$K_a$ of this group was determined to be 5.9 or 6.0 from the two respective sets of resonances (Figure 2, Table 1).

It was also apparent from examination of the alanine region of CTCOSY spectra that two alanine groups exhibited a sudden change in their chemical shifts at pH* greater than 6.4. These groups are AlaD and AlaE, which are not yet specifically assigned. The AlaE cross-peak changes such that it finally overlaps with Leu70, as indicated by doubling of this cross-peak volume at high pH*. In a study of a PROXYL maleimide-modified A67C mutant protein (Girvin & Fillingame, 1995), the proton resonances of AlaB and AlaC were broadened by the paramagnetic nitroxide, but resonances of AlaD and AlaE were not affected. These results indicate that AlaB and AlaC are at positions 20 and 21 and AlaD and AlaE at positions 39 and 40, respectively. The changes in chemical shifts of Ala39 and Ala40 at high pH* therefore suggest a more global conformational change in this region resulting from the deprotonation of the Asp61 carboxyl group (see additional results and discussion below).

Table 2: Summary of Carboxyl p$K_a$* Values in Gly-Gly-Asp-Ala Peptide

| | $^2H_2O$ Solvent | | deuterated C/M/W solvent | |
|---|---|---|---|---|
| group titrated | $H^\alpha$ | $H^\beta$ | $H^\alpha$ | $H^\beta$ |
| Asp3 $\beta$-carboxyl | nd[a] | 4.0, 4.0[b] | nd[a] | 5.5, 5.4[b] |
| Ala4 $\alpha$-carboxyl | 3.7 | 3.7 | 5.2 | 5.1 |

[a] Not determined due to the overlap with HDO resonance. [b] This value is for the second $H^\beta$ resonance.

*p$K_a$ of Arg and Lys Residues in Wild Type (A24D61) Subunit c.* There are three positively charged groups in the subunit *c* protein: Lys34, Arg41, and Arg50. There were no significant changes in the chemical shifts of these groups during the pH* titration, thereby indicating that they may have a p$K_a$ > 8.0.

*Expected Changes in p$K_a$ with Solvent.* In order to compare the p$K_a$ value of an aspartyl carboxyl in aqueous solution to that in C/M/W solvent, we have examined a model peptide, Gly-Gly-Asp-Ala. The p$K_a$ values for $\beta$ protons of Asp3 and the carboxyl terminus of Ala4 in C/M/W solvent were 5.4 and 5.1, respectively (Table 2), i.e. similar to those for Asp7, Asp44, and Ala79 in subunit *c*. These p$K_a$ values were approximately 1.5 pH* units higher in C/M/W solvent than in aqueous solvent (Table 2).

*D24D61 Mutant Subunit c.* We have analyzed the D24D61 mutant subunit *c* which contains Asp residues at both positions 24 and 61. The *in vivo* function in this mutant is pH dependent, and the carboxyl groups are predicted to have p$K_a$ values in the range of 7.4 (Zhang & Fillingame, 1994). A new cross-peak in the Asp/Asn region of CTCOSY spectra was assigned to Asp24 (see supporting information); it was clearly distinguished from that of Asp61 and the other Asp at all pH examined. The chemical shifts of $H^\alpha$-$H^\beta$ cross-peaks for both Asp24 and Asp61 begin to change at pH* 6.35. Asp24 titrates with a p$K_a$ value of 7.1, and Asp61 titrates with a p$K_a$ value of 6.9 (Figure 5). The changes in chemical shifts of Asp7 and Asp44 with pH* were similar to those in the wild type protein; i.e. these residues titrate with p$K_a$ values of 5.4 and 5.6, respectively (Figure 5). Ala79 in D24D61 mutant protein also exhibited a titration behavior similar to that in the wild type protein with a p$K_a$ of 5.8 (Figure 5). Other changes in the Ala region are noted below. The p$K_a$ of Asp24 is similar to that predicted for Asp24 in a functional $F_o$ *in situ* (Zhang & Fillingame, 1994).

*D24N61 Mutant Subunit c.* When the Asp/Asn regions of the CTCOSY spectrums of mutant and wild type proteins were compared, the cross-peak attributed to Asp61 was missing in the D24N61 protein (see supporting information). It was concluded that a cross-peak attributed to Asn61 overlaps with AsnB since the volume of the AsnB cross-peak doubles. A new cross-peak, which was also observed in the D24D61 mutant protein, was assigned to Asp24.

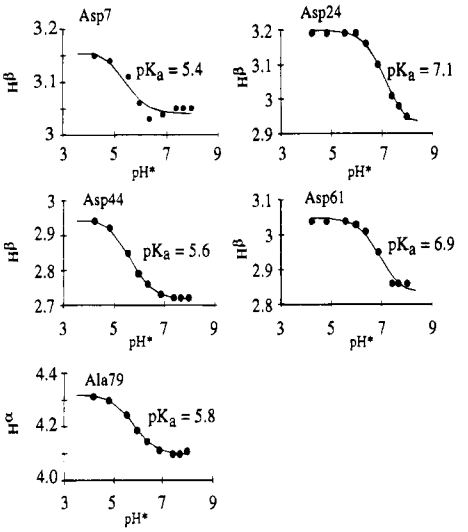


FIGURE 5: pH* titration of H$^\beta$ chemical shift for Asp residues in the D24D61 mutant subunit *c*. The titration of the C-terminal α-carboxyl of Ala79 is also shown using the change in chemical shift of H$^\alpha$. Solid lines are the theoretical curves, fit as described in Figure 2, and the solid circles are experimental values.

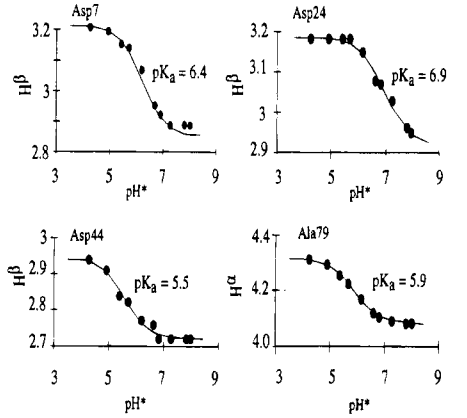


FIGURE 6: pH* titration of H$^\beta$ chemical shift for Asp residues in the D24N61 mutant subunit *c*. The titration of the C-terminal carboxyl of Ala79 is also shown using the change in chemical shift of H$^\alpha$. Solid lines are the theoretical curves, fit as described in Figure 2, and the solid circles are experimental values.

Asp44 titrates with a p$K_a$ similar to that in the wild type protein (Figure 6). However, Asp7 has a higher p$K_a$ value in the D24N61 mutant protein compared to the wild type protein. Asp24 titrates with a p$K_a$ of 6.9, which is similar to the p$K_a$ of Asp24 in the D24D61 protein. Ala79 titrates with a p$K_a$ of 5.9, i.e. similar to that in the D24D61 and wild type proteins (Figure 6).

*General Conformational Changes in Wild Type and Mutant Proteins Linked with Ionization of Essential Aspartyl.* We have compared the changes in chemical shifts in different sequentially assigned residues for wild type subunit *c* over two pH* ranges, i.e. between 4.7 and 5.7 and between 5.7 and 7.6 (Figure 7). Changes in the first range are local and can be attributed mainly to titration of the carboxyl residues of Glu2, Asp7, Glu37, and Asp44 and the C-terminal carboxyl group of Ala79. On the other hand, a new set of changes occur between pH* 5.7 and 7.6, including novel changes in the polar loop region and the N-terminus (Figure 7B). The changes in Pro43 and AlaE (Ala39 or Ala40) are particularly notable.[3] These changes can be attributed to the ionization of Asp61 and suggest a global change in the structure of the protein upon deprotonation of this residue. The conformational change in the polar loop region, i.e. residues 39−44, may be of functional relevance as is discussed below.

At pH* >6.2, the proton chemical shifts for AlaE (Ala39 or Ala40 in the polar loop) change significantly in both the D24D61 and D24N61 mutant proteins; i.e. the changes are similar to those seen in the wild type protein (Figure 7). The changes in AlaE chemical shifts thus seem to follow ionization of either Asp61 or Asp24 and may reflect similar conformational changes related to coupling. However, the large change in the α proton chemical shift of Pro43 exhibited by the wild type protein is not observed in the two mutant proteins. This may indicate subtle differences in the conformational changes transmitted to the polar loop region.

The D24N61 mutant protein shows a larger change in the N-terminal region (i.e. for AsnA and Asp7 residues) over a pH* range of 4.9−7.8 compared to the wild type protein. The chemical shift of the α proton of AsnA in the D24N61 mutant changes from pH* 6.2 to pH* 7.8 by about 0.1 ppm (see supporting information), but does not change appreciably in the wild type subunit *c* (Figure 1). In addition, the p$K_a$ of Asp7 is increased to 6.4 in the D24N61 mutant protein versus 5.4 in the wild type protein (compare Figures 2 and 6). The conformations of the N-terminal region of the D24N61 protein and wild type protein therefore appear to differ once Asp24 or Asp61 ionize.

## DISCUSSION

The p$K_a$ of the β-carboxyl group of Asp61 of subunit *c* is of interest since protonation/deprotonation of this carboxyl group is thought to mediate $F_o$-catalyzed proton translocation (Fillingame, 1990). The known properties of purified subunit *c* in C/M/W solvent, including the chemical reactivity of Asp 61 with DCCD, closely mimic those predicted for subunit *c* in the $F_o$ complex (Girvin & Fillingame, 1993, 1994, 1995). The p$K_a$ of Asp61 in this solvent may therefore prove to be of functional relevance. In the wild type (A24D61) protein, Asp61 had a p$K_a$* of 7.1, which was 1.5 pH units higher than the p$K_a$* of the two other Asp residues in the protein. The Asp61 p$K_a$* was also 1.5 units higher than the p$K_a$* measured for an Asp residue in a model tetrapeptide dissolved in C/M/W solvent. The studies with this peptide also suggest that the p$K_a$* of an exposed, Asp side chain carboxyl should rise from 4.0 to 5.5 on transfer from $^2H_2O$ to deuterated C/M/W solvent. This is expected due to the decrease in the dielectric constant for the solvent. Finally, in the D24N61 double mutant, the functional Asp24 also demonstrated an abnormally high p$K_a$* of 6.9. In summary, the functional carboxyl group appears to lie in an unusual environment when the protein folds in C/M/W solvent.

The p$K_a$ values of the carboxyl groups in isolated subunit *c* appear to be solvent dependent. Moody et al. (1987) concluded that Asp and Glu residues have p$K_a$ values higher than 9.0 in a $CHCl_3/CH_3OH$ (1:1 or 2:1) solvent mixture.

[3] There are three Pro residues in subunit *c*, two of which (Pro43 and Pro47) are not specifically assigned (Girvin & Fillingame, 1993). The residues are both located in the polar loop region. In studies of the Q42E mutant (Assadi, 1994), the chemical shifts of ProB were the only ones to change. Pro43 is the best candidate for ProB cross-peak since it is the closest to the site of the Q42E substitution; it is the resonance identified in Figure 7.

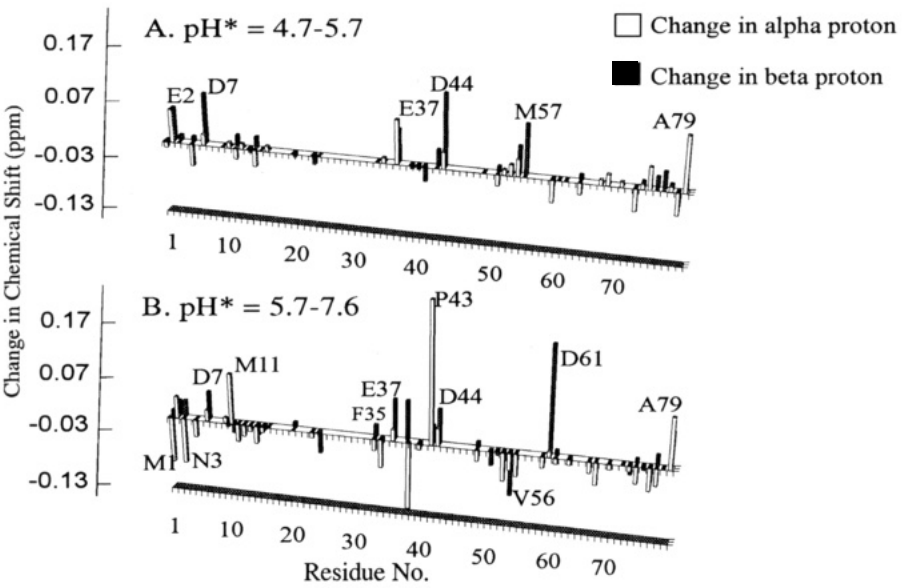


FIGURE 7: pH* dependence of the $H^{\alpha}$ and $H^{\beta}$ chemical shifts in sequentially assigned residues of wild type subunit *c*. The differences in chemical shifts are taken between pH* 4.7 and 5.7 (A) and between pH* 5.7 and 7.6 (B).

The lack of water in this solvent system may prevent ionization. In another study, using trifluoroethanol as solvent, Norwood et al. (1992) concluded that the three Asp and two Glu residues all ionize with p$K_a$ values < 5.5. In our studies in C/M/W solvent, the p$K_a$ of Asp61 in the wild type protein is significantly higher than that of any of the other carboxyl groups in the protein. The organic solvent mixture composed of $C^2HCl_3/C^2H_3O^2H/^2H_2O$ (4:4:1) made 50 mM in NaCl may provide an environment that promotes folding in a manner similar to that occurring in the membrane. Clearly, the structure of the protein is likely to vary, depending upon the solvent selected.

In order to rationalize the functional activity of the D24G61 and D24N61 subunit *c* mutants, Asp61 and Ala24 were hypothesized to lie at approximately the same position within the membrane and the two helices predicted to interact as a unit to which the essential carboxyl was anchored (Miller et al., 1990; Zhang & Fillingame, 1994). It then follows that the environment around the essential carboxyl in the D24N61 mutant protein should be similar to that in wild type protein, and this prediction is verified by the high p$K_a$ of Asp24 in the isolated D24N61 mutant protein. The conformation of the N-terminal region of D24N61 protein does appear to differ from that of the wild type protein, following ionization of Asp24 or Asp61. In the D24N61 protein, this difference is indicated by larger changes in the chemical shift of AsnA (Asn3 or Asn5) at high versus low pH and a higher p$K_a$ for Asp7. The D24N61 and wild type proteins do show an interesting difference *in situ* which may be related to these differences in structure. On removal of $F_1$, the wild type $F_o$ functions as a passive $H^+$ translocator. This passive, $H^+$ translocation activity is not observed with the D24N61 $F_o$, presumably because its $F_o$ is structurally less stable than wild type $F_o$ (Zhang & Fillingame, 1994). On the other hand, the D24N61 $F_1F_o$ complex shows substantial ATPase-coupled $H^+$ translocation activity, although the activity is less than with wild type $F_1F_o$.

In the case of the D24D61 mutant, both ATP-coupled proton translocation by membranes *in vitro* and the growth of cells were pH dependent; function was lost and growth inhibited as the pH was raised from pH 7 to 8 (Zhang & Fillingame, 1994). The protonation of Asp24 was postulated to be necessary in order for Asp61 to function in a catalytic cycle of protonation/deprotonation, and the apparent p$K_a$ of Asp24 was suggested to lie between pH 7 and 8. The high p$K_a$ measured here for the Asp24 in the isolated D24D61 mutant protein is consistent with the prediction from the functional studies.

Previous NMR studies provide some information on the structure of subunit *c* in the region around Asp61. In Girvin and Fillingame (1995), the structure in the region of the protein including residues 10−25 and 60−77 was modeled using distance constraints derived from spin-label difference spectra of the PROXYL-malemide-modified Val67 → Cys mutant subunit *c* protein. The Asp61 carboxyl was predicted to lie within van der Waals contact of the Ala24 $\beta$-carbon in a pocket bordered partially by the Met65, Ile63, and Val60 side chains. Previous studies of the NCCD-modified subunit had suggested a close proximity of the Ile28 and Met57 side chains to the aspartyl pocket (Girvin & Fillingame, 1994). The p$K_a$ of a carboxyl buried in such a hydrophobic cavity would be expected to be higher than that of a carboxyl exposed to solvent and may explain the unusual p$K_a$ observed here. The exact structure of the pocket will not be known until ongoing efforts to solve the structure of the entire protein are complete. We note that subunit *c* is predicted to interconvert between a high-p$K_a$ form and a low-p$K_a$ form during the active proton-pumping cycle that is coupled to ATP hydrolysis (Fillingame, 1990). The conformation of isolated subunit *c* being solved by NMR will most likely correspond to the high-p$K_a$ form of the protein. A high-p$K_a$ form of the protein is also likely to be the form that mediates passive $H^+$ transport by $F_o$ in the absence of $F_1$ (Okamoto et al., 1977; Kluge & Dimroth, 1993).

The large changes in the chemical shift of AlaE (Ala39 or Ala40) and Pro43 in the polar loop at pH* >6 must result from deprotonation of Asp61, where ionization must lead to conformational changes which ultimately affect the structure of the loop region. The structural changes will be of functional interest since protonation/deprotonation of the Asp61 is thought to be coupled to conformational changes in the polar loop which are ultimately propagated to $F_1$ and

linked to ATP synthesis (Mosher et al., 1985; Fraga et al., 1994; Zhang et al., 1994).

*Conclusion.* The pH titration of wild type and mutant subunit *c* in C/M/W solvent indicates that the protein folds to create a unique environment around the functional, $H^+$-translocating carboxyl group. This study provides further evidence that the structure of subunit *c* in C/M/W solvent closely mimics that in native $F_o$.

## ACKNOWLEDGMENT

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## SUPPORTING INFORMATION AVAILABLE

A figure showing expansions of the Ala $H^{\alpha}-H^{\beta}$ region of CTCOSY spectra for wild type subunit *c* at varying pH*, a figure showing expansions of the Asp/Asn $H^{\alpha}-H^{\beta}$ region of CTCOSY spectra for the D24D61 mutant protein at varying pH*, and a figure showing expansions of the Asp/Asn $H^{\alpha}-H^{\beta}$ region of CTCOSY spectra for the D24N61 mutant protein at varying pH* (4 pages). Ordering information is given on any current masthead page.

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