Selective Detection of Mobile Amino Acid Resonances of Chloroplast ATP Synthase by ^{1}H Spin-Echo NMR†

R. R. Sharp*,‡ and W. D. Frasch§

Department of Cellular and Molecular Biology and Department of Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

Received January 4, 1985

ABSTRACT: High-resolution proton spin-echo NMR spectra have been recorded for the solubilized chloroplast coupling factor 1 (CF_1). A Hahn spin-echo sequence has been used to suppress the intense unresolved background signal that arises from protons in relatively structured regions of the enzyme, thereby permitting selective detection of the relatively small subset of protons which comprise the more mobile resonances on the enzyme. The 18-ms spin-echo spectrum consists of resonances which arise from approximately 120 protons (about 20 amino acids) per molecule of CF_1. Characteristic line widths are in the range 1–10 Hz (T_2 in the range 30–300 ms), which indicates a reorientational correlation time about 10^2 times shorter than that of the majority of proton resonances on CF_1. Several peaks exhibit resolved multiplet structures and show J modulation in spin-echo spectra obtained at long τ. Specific resonances due to aspartate β-methylene protons, glutamate γ-methylene protons, lysine ϵ-methylene protons, and tyrosine aromatic protons have been assigned on the basis of (1) studies of the effects of selective covalent modification of the enzyme, (2) comparisons of observed chemical shifts with those of short reference peptides in aqueous media, and (3) spin-coupled multiplet structure. Chemical shifts of resonances assigned by covalent modification techniques correspond closely to values characteristic of aqueous media. Quantitative measurements indicate involvement of two to three lysines, one to two aspartates, one glutamate, and zero cysteine. The 18-ms spectra also contain resolved intensity from α-methine backbone protons in the region 3.9–4.6 ppm, suggesting the involvement of one or more random-coil peptide segments which extend into the aqueous phase.

High-resolution ^{1}H NMR spectroscopy has provided detailed structural and dynamical information for several proteins of low or moderate molecular weight. The most complete spectral assignments have been obtained for proteins for which the X-ray structure is known; among these, lysozyme (Campbell et al., 1975a–d) and bovine pancreatic trypsin inhibitor (Dubs et al., 1979; Wüthrich, 1980, 1981) have been intensively studied. More recently, the emerging technique of two-dimensional (2-D)[1] NMR has provided a systematic approach to problems of spectral assigmment and structure (Wüthrich, 1983; Billeter et al., 1982; Wagner & Wüthrich, 1982; Wider et al., 1982).

The most serious limitation inherent in the use of NMR to study enzyme structure is the large dipolar line width which results from long reorientational correlation times. For proteins with a molecular weight of approximately 10^5 or greater, dipolar line broadening obscures most of the structure in proton spectra, even at magnetic field strengths in excess of 10 T. However, a small subset of amino acid side chains often experiences atypically high reorientational mobility, even in very large proteins. The present report shows that a subset of predominantly hydrophilic amino acids in fact contributes resolved spectral structure to the proton NMR spectrum of a large, multisubunit enzyme, solubilized chloroplast coupling factor 1 (CF_1).

The proton-transporting ATP synthase is ubiquitous to organisms in the animal and plant kingdom where it catalyzes the synthesis of ATP. In plants, the storage of electromagnetic energy from light during photosynthesis is culminated by formation of the terminal phosphate bond of ATP. The ATP synthase consists of two major components: first, an intrinsic membrane protein complex, chloroplast coupling factor 0 (CF_0), which is believed to act as a proton channel; and second, an extrinsic membrane protein complex, CF_1, which contains the active site for ATP synthesis (Nelson, 1981). Solubilized CF_1 contains five different polypeptides which are believed to have a stoichiometry of α_3, β_3, γ, δ, ϵ and a total molecular weight of 400K–407K (Moroney et al., 1983).

We describe here the use of ^{1}H spin-echo NMR to suppress selectively the background of the unresolved spectral structure of soluble CF_1 and thereby to produce spectra in which the resolved resonance of mobile side chains can be studied in greater detail. A similar technique has been used by Brown et al. (1977) to detect soluble metabolites in intact erythrocytes. The spin-echo sequence circumvents problems of limited dynamic range of the digitizer and produces proton spectra containing resolved structure that are of considerably higher quality than is observed without the spin-echo technique. Identification of several of the peaks in the SE spectrum of CF_1 was made (1) by comparison to chemical shift values of random-coil polypeptides, (2) by changes in the SE spectrum

† This work was supported by grants from the U.S. Department of Agriculture (83-CRCR-1-1339) to W.D.F. and R.R.S. and from the Rackham Foundation and the Phoenix Memorial Research Foundation to W.D.F.

‡ Department of Chemistry.
§ Department of Cellular and Molecular Biology.

[1] Abbreviations: AMPPNP, 5′-adenylyl β-imidodiphosphate; SE, spin-echo; SED, spin-echo difference; CF_1, chloroplast coupling factor 1; CF_0, coupling factor 0; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; FID, free induction decay; DTT, dithiothreitol; WRK, Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate); HPLC, high-performance liquid chromatography; DCCD, dicyclohexylcarbodiimide; PCMB, *p*-(chloromercuri)benzenesulfonate; 2-D, two-dimensional; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; MA, maleic anhydride.

0006-2960/85/0424-5449$01.50/0

resulting from modification of specific side chains of the protein by covalent modifying reagents, and (3) by analysis of multiplet structure and J modulation.

Experimental Procedures

Isolation of CF_1. Chloroplast CF_1 was solubilized from the thylakoid membranes of spinach (Frasch & Selman, 1981) by washing the membranes in 4 L of buffer with low ionic strength. To purify the crude extract, the protein was bound to DEAE-Sephadex A50 as described by Binder et al. (1978) and eluted from the gel with the standard buffer (2 mM EDTA, 1 mM ATP, and 20 mM Tris-HCl, pH 7.1) containing 300 mM $(NH_4)_2SO_4$. This chromatographic step served to concentrate the protein and to remove traces of membranes. The protein was concentrated further by precipitation with 50% $(NH_4)_2SO_4$. After resolubilization of the protein and desalting by chromatography on Sephadex G-25, the desalted protein was purified by anion-exchange high-performance liquid chromatography using a 150 mm × 21.5 mm Bio-Sil TSK IEX-543 DEAE column.

The HPLC column was preequilibrated in the standard buffer containing 135 mM $(NH_4)_2SO_4$ without ATP. The elution profile of the column was determined by raising the ammonium sulfate concentration of this buffer in the following manner. At a rate of 10 mL/min, the salt concentration was raised from 13 to 175 mM in a linear gradient over a period of 2.5 min, then from 175 to 350 mM in a linear gradient during the next 5 min, and finally from 350 up to 600 mM in a linear gradient of 5-min duration. Elution of the protein was monitored at 280 nm and eluted in the 175–350 mM gradient. The purified protein was stored as an ammonium sulfate precipitate in 2 mM ATP, 1 mM EDTA, and 40 mM Tricine, pH 8.0 at 4 °C. Measured ATPase activity was typically 24 μmol mg^{-1} h^{-1} at pH 8.0.

Preparation of Protein Samples. Just prior to use, the protein was pelleted, the supernatant decanted, and the pellet solubilized in 5 mL of 40 mM $BO_4 \cdot 10D_2O$/DCl buffer, pH 8.7 in D_2O (99.8% D). The ATPase was activated by adding dithiothreitol (DTT) as a solid to the protein solution to a final concentration of 50 mM. After a 90-min incubation in DTT, during which the protein solution was concentrated to 0.5 mL by pressure dialysis, the protein was passed through a column of Sephadex G-50-80 (0.5 × 15 cm). Preparation of the Sephadex involved exhaustive prewashing with glass-distilled water, equilibration with D_2O, lyophilization to dryness, and final equilibration with borate buffer. The activated enzyme was further washed by two cycles of dilution in borate/D_2O buffer and reconcentrated by pressure dialysis using an Amicon XM-100 membrane. The resulting sample (0.6 mL) was further deuterated by several cycles involving addition of highly pure D_2O (99.96%) followed by evaporation to the original volume under a gentle stream of nitrogen gas, during which the solution was maintained at 30 °C.

NMR Measurements. High-resolution ^1H NMR spectra were obtained at 37 ± 1 °C by using a Bruker WM-360 spectrometer. Spin-echo difference spectra revealed slow changes in side-chain mobilities during the first hour of incubation at 37 °C, but difference spectra obtained subsequently over a period of several hours nulled almost completely (see Figure 4). Enzyme activity dropped approximately 15% during the first hour of incubation at 37 °C but was relatively stable for a period of 4 h thereafter. The residual ^2HHO peak of the solvent was suppressed by inversion–recovery using a selective 180° pulse of 12-ms duration. A spin-echo sequence (90°–τ–180°–τ–accumulate) was used where indicated and was initiated at the null of the solvent peak. Typical spin-echo spectra comprised 1500 transients of 8192 data points and a spectral bandwidth of 8000 Hz. Phase cycling was not used in the spin-echo sequences: for this reason, the carrier frequency was placed at the low-field end of the spectrum (9 ppm) to displace quadratic images of the strongest peaks outside the spectral region of interest. The total accumulation time was 12–15 min per pulse spacing.

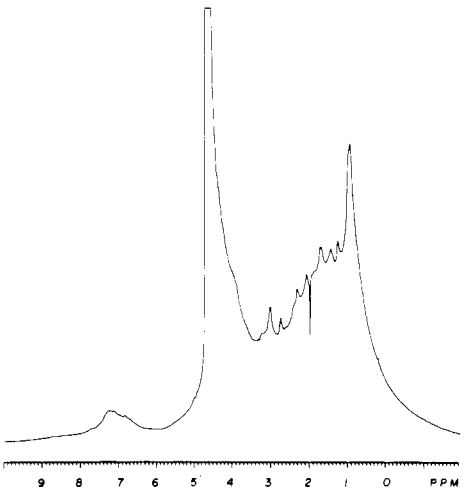

FIGURE 1: 360-MHz proton NMR spectrum (non-spin-echo) of CF_1 activated by dithiothreitol. The soluble enzyme (18 mg/mL in deuterated borate buffer, pH* 8.7) was observed at 37 °C at the null of the solvent peak, which was suppressed by a selective 180° pulse. The reference is internal DSS. The spectrum is an accumulation of 500 transients subjected to apodization equivalent to 2-Hz line broadening.

The integral of the spin-echo spectrum was quantitated by comparison with the peak integral of the residual solvent proton resonance in the same sample, recorded without solvent suppression. A suitable reference peak was recorded by offsetting the carrier to place the ^1HDO peak to the low-field end of the spectrum (13 ppm) and collecting a few scans without solvent suppression and with a sufficient delay to avoid saturation. This reference free induction decay was coadded to and transformed with the analytical spectrum. The solvent proton concentration contributing to the reference peak was measured by direct comparison of the reference integral, measured under continuous-wave conditions, to that of a sample of pure water.

Results

Purified CF_1 is comprised of 3931 amino acids and has a total molecular weight of approximately 400 000 (Moroney et al., 1983). It appears in electron micrographs as an ovoid particle of dimensions 80 × 110 × 120 Å (Amzel & Pederson, 1978; Amzel et al., 1982). For a particle of this size in aqueous solution, the Stokes–Einstein reorientational correlation time is approximately 0.10 μs at 37 °C, which implies a dipolar line width of the order of 400 Hz for methylene protons which are constrained to reorient with the protein. (This estimate includes only the geminal proton coupling and neglects effects of internal degrees of motional freedom.)

The 360-MHz ^1H NMR spectrum of CF_1, recorded by Fourier transformation of the free induction decay following a 40° pulse, is shown in Figure 1. The major portion of the observed intensity is unresolved and contributes as four very broad bands arising from saturated methyl, methylene, and methine protons and from aromatic protons. Superimposed on this unresolved background are several more highly resolved peaks.

To suppress unresolved background intensity and thereby enhance the relative contribution due to motionally narrowed

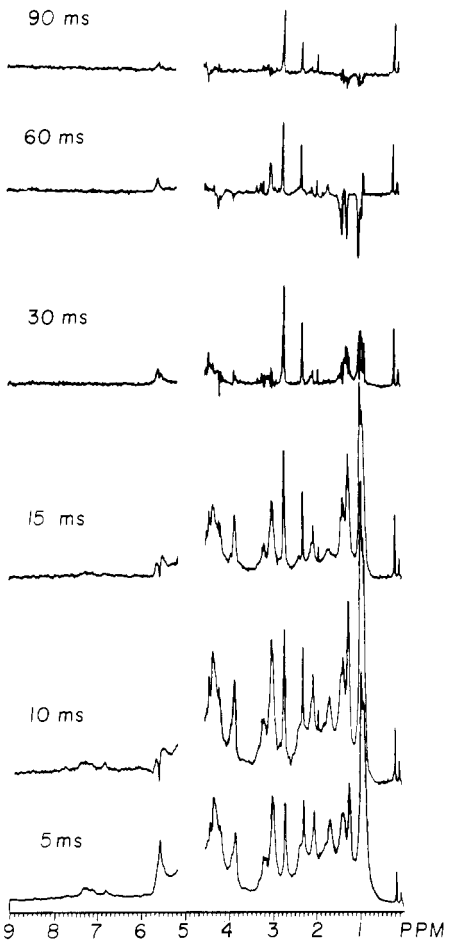

FIGURE 2: Spin-echo spectra of DTT-activated CF_1 at various 90°–180° pulse spacings. Each spectrum represents 1500 transients subjected to exponential apodization equivalent to 2-Hz line broadening. The sample contained 25 mg/mL CF_1 in 40 mM borate buffer, pH* 8.7. The pulse spacing (τ) of each spectrum is indicated.

peaks, spin-echo spectra were accumulated subsequently. A series of spin-echo spectra obtained at increasing 90°–180° interpulse spacings is shown in Figure 2. In the absence of significant J modulation, spin-echo spectra discriminate among component peaks of the high-resolution spectrum on the basis of T_2 differences, with peaks broader than approximately $\Delta\nu = (\pi T_2)^{-1}$ being effectively suppressed. This kind of discrimination is evident in Figure 2. A pulse spacing of 5 ms, which provides a filter for resonances broader than about 30 Hz, removes more than 90% of the total magnetization.

Lengthening the interpulse spacing progressively removes magnetization from the spectrum, but significant intensity remains even at quite long pulse spacings, $90 \geq \tau \geq 15$ ms ($\Delta\nu_{1/2} \leq 10$ Hz). The narrowest peaks observed in spin-echo spectra at long τ have intrinsic line widths in the range 1–5 Hz. Spectra at 90 ms contain a series of five sharp, apparent singlets at 2.70, 2.28, 1.89, 0.18, and 0.02 ppm. Each of these peaks appears to arise from a single amino acid side chain (or from chemically equivalent side chains). Figure 3 shows the T_2 relaxation of these peaks, which follows simple exponential behavior for $\tau \geq 15$ ms. The calculated T_2's range from 85 ms (2.28 ppm) to greater than 200 ms (0.18 ppm). In spin-echo spectra obtained at pulse spacings of 30–90 ms, spin-coupled multiplets showing pronounced effects of J modulation are present at ca. 4.3, 3.9, 3.2, 3.0, 1.34, 1.18, and 0.9 ppm.

The presence of such narrow resonances was surprising and caused us to suspect interference by low molecular weight contaminants. To test this possibility, the protein was washed

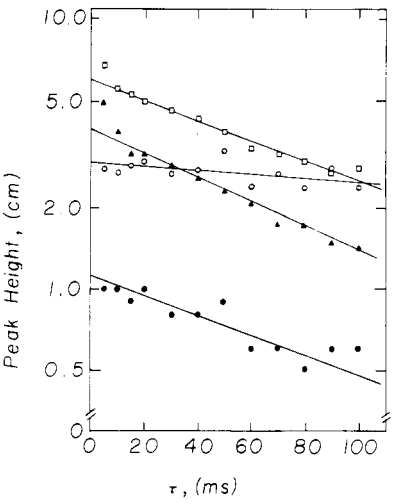

FIGURE 3: T_2 relaxation of four of the resolved resonances in the spin-echo NMR spectra of DTT-activated CF_1. Peak heights for the peaks at 2.7 (squares), 2.25 (triangles), 0.2 (open circles), and 0.1 ppm (closed circles) were plotted as a function of the pulse spacing, τ, from the data in Figure 2.

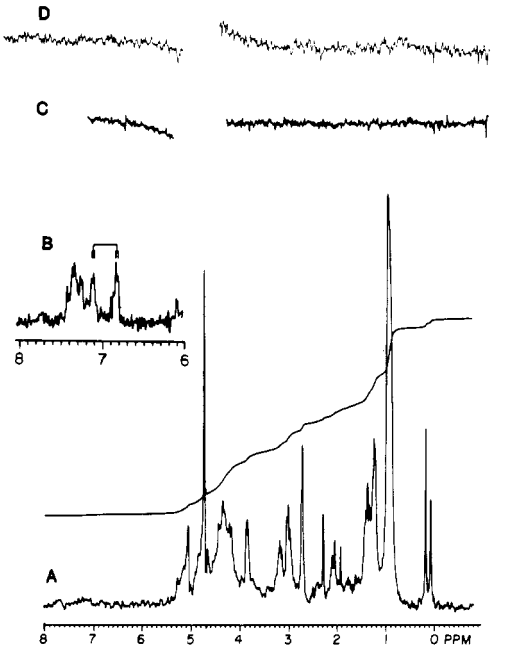

FIGURE 4: Quantitation of the spin-echo spectrum of DTT-activated CF_1 with a pulse spacing of 18 ms. The effective concentration of protons contributing to the spectrum was calculated as described under Experimental Procedures. (A) Integrated spectrum. (B) The 6-ms spin-echo spectrum of the aromatic region of CF_1 at 45 °C. A shifted Gaussian apodization function (Lindon & Ferrige, 1979) has been applied for resolution enhancement. The AA'XX' multiplets of tyrosine are indicated. (C) Effluent following pressure dialysis with an Amicon XM-100 membrane of the solution of CF_1 which had been used to obtain spectum A. (D) Spin-echo difference spectrum formed by subtracting spectra taken 3 h apart.

repeatedly (8 times) with borate buffer on an Amicon XM-100 filter (molecular weight cutoff ≈105 000) by pressure dialysis, and the effluent and residuum were observed by NMR. The narrow peaks remained with the protein, and the effluent was devoid of NMR-visible resonances (Figure 4C). In a previous communication, we reported spectra which contained peaks due to a dissociated sugars from the Sephadex G-50 used in the protein purification. In the present work, these contaminants were eliminated very effectively through the use of gentle lyophilization procedures to deuterate the Sephadex and by routine use of repeated pressure dialysis steps to purify the

Table I: Estimated Proton Concentrations Corresponding to Resolved Peaks in the 18-ms Spectrum of Figure 4[a]

chemical shift (ppm)	assignment	$^1H/CF_1$[b] (mol/mol)	$^1H/CF_1$[c] (mol/mol)
2.70	Asp β-CH_2	2.8	3.3
3.0	Lys ϵ-CH_2	3.5	4.6
2.3	Glu γ-CH_2	1.0	1.25
0.18	unassigned CH_3	3.4	3.7
0.0–8.0	total ^1H	85	120

[a] Concentrations were calculated from the spectrum integral by using the integral of the residual solvent ^2HHO peak as a quantitative reference peak as described under Experimental Procedures. [b] Uncorrected for T_2 relaxation. [c] Corrected for relaxation.

protein. All of the remaining structure in our current spin-echo spectra (e.g., Figure 2) survives both HPLC purification and Amicon filtration and is ascribable to amino acids (or possibly sugars) covalently bound to CF_1.

The spin-echo spectra have been quantitated by comparison of the spectrum integral with that of the solvent HDO peak of the sample, which provided a convenient reference peak of known proton concentration (see Experimental Procedures). An integration of the spin-echo spectrum at 18 ms is shown in Figure 4. This spectrum corresponds to a total proton concentration of 4.14 mM or 85 mol of ^1H per mole of CF_1. After accounting for the T_2 decay, we estimate that the parent intensity corresponds to about 120 mol of protons per mole of CF_1 or approximately 20 amino acids per molecule of CF_1. Estimated proton concentrations corresponding to the major peaks in Figure 4 are given in Table I.

Peak Assignments. Preliminary spectral assignments were obtained by comparison of the spectra with reference chemical shifts of protons on short random-coil peptides dissolved in neutral aqueous media (Bundi & Wüthrich, 1979). Several of the prominent peaks in the 18-ms spin-echo spectrum correspond closely to the chemical shift values of side-chain resonances of charged amino acids in the reference peptides. Tentative assignments of the major peaks have been made on the basis of chemical shift information and multiplet structure. These assignments have been confirmed in several cases by covalent modification of the protein.

Tyrosine and Phenylalanine. The pair of peaks at 6.83 and 7.13 ppm in the 6-ms spin-echo spectrum (Figure 4B) corresponds to the $\alpha\alpha'XX'$ pattern of tyrosine aromatic protons. After resolution enhancement, the A and X resonances exhibit multiplet structure with a major coupling constant of J_{AX} = 8.5 Hz. The coupling constant and chemical shifts are almost identical with reported values for tyrosine in random-coil aqueous peptides (Bundi & Wüthrich, 1979). The three peaks at 7.2–7.4 ppm are assigned to phenylalanine aromatic protons, which resonate at this position in an aqueous environment.

Carboxyls. Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3-sulfonate) reacts with accessible carboxylate side chains on CF_1 to give enol esters (Arana & Vallehos, 1980, 1981). Figure 5 shows the spin-echo difference (SED) spectrum of CF_1 formed by subtracting the spectrum of protein modified by WRK from the spectrum of the unmodified protein. The large negative peaks in the SED spectrum at 1.15 and 3.30 ppm result from the methyl and methylene protons, respectively, of WRK which are only present in the sample of modified protein.

Changes produced in the spectrum of CF_1 are subtle and highly specific. Positive difference peaks, which reflect side-chain immobilization in the modified protein, occur at 2.70, 2.18, and 1.86 ppm and correspond closely to the β- and γ-methylene resonances of aspartate and glutamate in model aqueous peptides. The sensitivity of these peaks in WRK

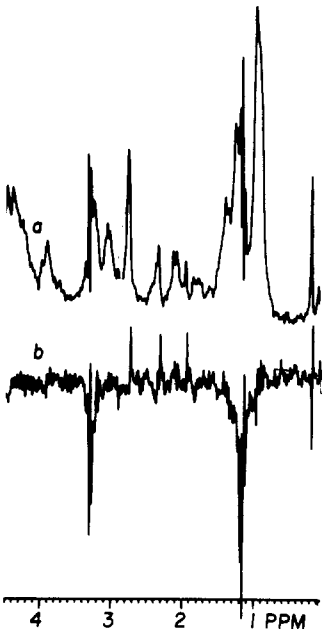

FIGURE 5: Effect of covalent modification by Woodward's reagent K on the spin-echo spectrum of DTT-activated CF_1. (a) Spin-echo spectrum at τ = 18 ms prior to modification of the protein. (b) Spin-echo difference spectrum obtained by subtraction of the spectrum of the modified protein from that of the unmodified protein.

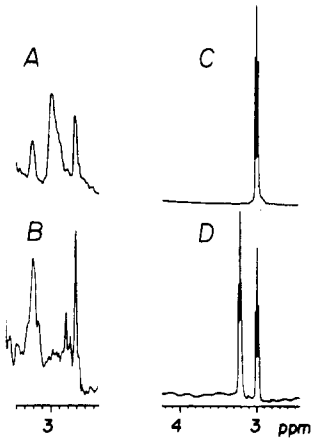

FIGURE 6: Effect of covalent modification by maleic anhydride on the spin-echo spectrum of DTT-activated CF_1. Chemical shift of the ϵ-methylene resonance of the reference compound, α-aminocaproic acid, before (C) and after (D) incompletely reacting with maleic anhydride. Spin-echo spectra (τ = 18 ms) of CF_1 before (A) and after (B) modification with maleic anhydride.

supports a corresponding assignment in the 18-ms SE spectrum of CF_1. The absence of spin–spin splitting in these resonances evidently reflects decoupling of the multiplet substructure due to relatively efficient spin relaxation in this system.

Lysine. Multiplets at 3.0, 1.68, and 1.41 ppm are assigned to the ϵ-, δ-, and γ-CH_2 groups of lysine, respectively. The identity of the peak at 3.0 ppm has been confirmed by monitoring the effect of maleic anhydride on the 18-ms SE spectrum, a reagent which converts primary amines to amides at pH 9.0. This treatment results in a reversal of the charge of these side chains. Experiments with the model compound α-aminocaproic acid showed that MA modification produces a downfield titration shift from 3.0 to 3.2 ppm (in the ϵ-CH_2 resonance (Figure 6).

Modification of CF_1 with maleic anhydride produced the predicted displacement of the multiplet at 3.0–3.2 ppm (Figure 6), confirming the assignment of this peak. In addition to shifting the ϵ-lysine resonance, maleic anhydride modification

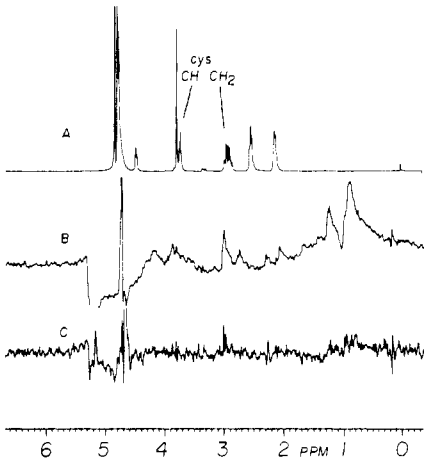

FIGURE 7: Effect of covalent modification by PCMB on the spin-echo spectrum of DTT-activated CF_1. (A) Spin-echo difference spectrum of the model compound dithiothreitol before vs. after modification with PCMB. (B and C) Spin-echo difference spectrum before vs. after modification of CF_1 with PCMB using spectra obtained at a pulse spacing of 4 (B) and 18 ms (C).

produced a pronounced increase in the line widths of most of the other resonances in the spectrum. This behavior is in marked contrast to that resulting from modification of carboxyls with WRK, which causes highly specific perturbations of the spectrum. Apparently, the alteration of electric charge which results from maleic anhydride modification causes a mobility decrease experienced in common by several amino acids that contribute to the spin-echo spectrum.

Cysteine. The possibility that cysteine contributes to the spin-echo spectrum was examined by modification with *p*-(chloromercuri)benzenesulfonate (PCMB). Spin-echo difference spectra formed ±PCMB at two pulse spacings are shown in Figure 7A,B. PCMB produces very little perturbation of the spectrum at 18 ms, indicating that accessible cysteine contributes negligible intensity at this pulse spacing. Spin-echo spectra obtained at 4 ms do show a prominent resonance at 3.0 ppm, indicating participation of a Cys β-CH_2 for which the chemical shift is not significantly perturbed from that of the aqueous amino acid. Smaller broad peaks are also observed at 2.67, 2.18, and 1.97 ppm, and in the methyl region at ca. 1.0 ppm. These peaks probably arise from neighboring amino acids that are motionally perturbed by cysteine modification.

Discussion

The present study has used the spin-echo sequence as a filter for selective observation of a small subset of more highly mobile amino acids on isolated CF_1. The use of spin-echoes to observe small populations of highly resolved resonances circumvents problems of dynamic range and receiver gain which otherwise severely lower the detection limits. The resonances observed in the 18-ms spin-echo spectra comprise 0.38% of the nonexchangeable protons on CF_1. These resonances were very poorly resolved or essentially undetectable in non-spin-echo spectra (compare, for example, the aromatic regions of Figures 1 and 4).

Spin-echo spectra permit an experimental resolution, on the basis of differing mobilities, of three groups of amino acids on CF_1. First, the bulk of the 3931 amino acid side chains of CF_1 has a reorientational mobility which is comparable to that of the enzyme complex as a whole. These resonances have line widths of several hundred hertz and contribute unresolved background intensity in the non-spin-echo spectrum (Figure 1). Second, spin-echo spectra obtained in the range of 3 ms $\leq \tau \leq$ 10 ms eliminate more than 90% of the unresolved background, revealing a number of partially resolved peaks with line widths ranging from 1 to 30 Hz (Figure 2). These spectra can be resolved fairly well in the aromatic region by using a resolution-enhancing window function (Figure 4B), but the upfield region of aliphatic protons is still obscured by overlap and contains peaks with line widths of the order of 20–30 Hz. The T_2 decay in this region is markedly nonlinear (Figure 3). The third group of amino acids contributing to the spectrum of CF_1 is observed at $\tau > 10$ ms. These side chains have intrinsic line widths in the range of 1–5 Hz and represent approximately 0.4% of the nonexchangeable protons on CF_1. This translates to approximately 120 protons or 20 amino acids per molecule of CF_1.

Several of the experiments described here suggest that some of the amino acids in this third subset of about 20 amino acids comprise an unstructured terminal segment of one of the subunits of CF_1. Evidence supporting this hypothesis includes the following: (i) the motional correlation times inferred from T_2's of protons visible in the 18-ms SE spectrum are some 2 orders of magnitude shorter than that of CF_1 as a whole; (ii) the transverse decay of these resonances follows simple exponential behavior at times longer than about 10 ms, indicating that individual peaks correspond to specific amino acid side chains; (iii) the spectral region from 4.0 to 5.0 ppm in the τ = 18 ms spectrum (Figure 4), which contains the resonances of α-methine protons of the protein backbone, is indicative of a highly mobile, random-coil segment [the lack of secondary structure in the peptide backbone is evident in the short correlation times (long T_2's) of the α-proton resonances; the chemical shifts of these peaks are also indicative of a random-coil structure (Jardetzky, 1970)]; (iv) the chemical shifts of assigned side-chain protons are quite close to values expected for short peptides in aqueous media; (v) when the charge of the lysines which contribute to the spectrum is reversed by modification, the mobility of several other amino acids in the highly mobile subset is dramatically decreased, suggesting their presence in a common chain.

The location of these NMR-visible amino acids with respect to the nucleotide binding site, which is generally believed to lie on the β subunit, is considered further in the following paper (Frasch & Sharp, 1985). It is shown there that carboxyl-bearing side chains are immobilized by nucleotide binding and lie within the dipolar sphere of influence of the tight Mn^{2+} binding site.

Registry No. ATP synthase, 37205-63-3; L-aspartic acid, 56-84-8; L-glutamic acid, 56-86-0; L-lysine, 56-87-1; L-tyrosine, 60-18-4.

References

Abragam, A. (1961) *The Principles of Nuclear Magnetism*, pp 58–63, Oxford University Press, Oxford.

Amzel, L. M., & Pederson, P. L. (1978) *J. Biol. Chem. 253*, 2067–2069.

Amzel, L. M., McKinney, M., Narayanan, P., & Pederson, P. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5852–5856.

Arana, J. L., & Vallejos, R. H. (1980) *FEBS Lett. 113*, 319–322.

Arana, J. L., & Vallejos, R. H. (1981) *FEBS Lett. 123*, 103–106.

Billeter, M., Braun, W., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 321–346.

Binder, A., Jagendorf, A., & Ngo, E. (1978) *J. Biol. Chem. 253*, 2094–3100.

Brown, F. F., Campbell, I. D., Kuchell, P. W., & Rabinstein, D. L. (1977) *FEBS Lett. 82*, 12–16.

Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 85–297.

Campbell, I. D., Dobson, C., & Williams, R. J. P. (1975a) *Proc. R. Soc. London, B 189*, 485–502.
Campbell, I. D., Dobson, C., & Williams, R. J. P. (1975b) *Proc. R. Soc. London, B 189*, 503–517.
Campbell, I. D., Dobson, C., & Williams, R. J. P. (1975c) *Proc. R. Soc. London, A 345*, 23–40.
Campbell, I. D., Dobson, C., & Williams, R. J. P. (1975d) *Proc. R. Soc. London, A 345*, 41–59.
Dubs, A., Wagner, G., & Wüthrich, K. (1979) *Biochim. Biophys. Acta 577*, 177–194.
Esch, F. S., Bohlen, P., Otsuka, A. A., Yoshida, M., & Allison, W. S. (1981) *J. Biol. Chem. 256*, 9084–9089.
Frasch, W. D., & Selman, B. (1981) *Biochemistry 20*, 3636–3643.
Frasch, W. D., & Sharp, R. R. (1984) *Adv. Photosynth. Res., Proc. Int. Congr. Photosynth., 6th 2*, 591–594.
Frasch, W. D., & Sharp, R. R. (1985) *Biochemistry* (following paper in this issue).
Lindon, J. C., & Ferrige, A. G. (1979) *J. Magn. Reson. 36*, 277–280.
Moroney, J. V., Lopresti, L., McEwen, B. F., McCarty, R. E., & Hammes, G. G. (1983) *FEBS Lett. 158*, 58–62.
Nelson, N. (1981) *Curr. Top. Bioenerg. 11*, 1–29.
Wagner, G., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 347–366.
Wider, G., Lee, K. H., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 367–388.
Wüthrich, K. (1980) *Front. Bioorg. Chem. Mol. Biol., Proc. Int. Symp.*, 161–168.
Wüthrich, K. (1981) *Macromol. Chem. Phys., Suppl. No. 5*, 234–252.
Wüthrich, K. (1983) *Biopolymers 22*, 131–128.

Nucleotide Binding to Chloroplast ATP Synthase: Effect on the Proton Spin-Echo NMR Spectrum†

W. D. Frasch*,‡ and R. R. Sharp§

Department of Cellular and Molecular Biology and Department of Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

Received January 4, 1985

ABSTRACT: Effects of nucleotide binding on the high-resolution proton spin-echo spectrum of chloroplast ATP synthase [coupling factor 1 (CF_1)] have been studied. Spin-echo difference spectra obtained at an 18-ms pulse spacing have been recorded ± stoichiometric amounts of 5′-adenylyl β-imidodiphosphate (AMPPNP), a nonhydrolyzable substrate analogue. Addition of Mg·AMPPNP to solutions of CF_1 causes a highly specific shortening of T_2 of two proton resonances at 2.70 and 2.29 ppm, which have previously been assigned to β- and γ-methylene protons of aspartate and glutamate, respectively, on the basis of studies of the effects of covalent modification of carboxyl-bearing side chains on CF_1. The observed T_2 shortening, which indicates decreased mobility of these side chains in the presence of nucleotide, results from nucleotide binding to a tight side ($K_d \approx 10^{-6}$ M) which is present at a mole ratio of 1 mol of nucleotide per mole of CF_1. Parallel experiments have also been conducted with the mangano-AMPPNP complex, which is paramagnetic and can produce additional relaxation enhancements of neighboring protons by means of the through-space nuclear dipole–electron dipole interaction. The effective range of this interaction in the present experiments is estimated to be at least 19 Å but no greater than 25 Å. From a comparison of relaxation enhancements produced by Mg·AMPPNP with those produced by Mn·AMPPNP, it is concluded that the majority of the resonances visible in the 18-ms spin-echo NMR spectrum lie outside the dipolar sphere of influence. The aspartate and glutamate resonances which are immobilized by Mg·AMPPNP binding do not coordinate directly to the metal ion but lie near the periphery of this sphere.

Coupling factor 1 (CF_1)[1] is the extrinsic membrane protein complex which contains the active site of the proton-translocating ATP synthase of the chloroplast thylakoid membrane. This enzyme mediates the transfer of free energy from the protonmotive force produced by photosynthetic electron transport to the synthesis of the terminal phosphate bond of ATP. CF_1 is composed of five distinct subunits, the apparent stoichiometry of which is $\alpha_3, \beta_3, \gamma, \delta, \epsilon$ (Dunn & Hepple, 1981; Penefsky, 1979; Merchant et al., 1983). The overall molecular weight of the extrinsic protein complex is 400K–407K (Moroney et al., 1983). Evidence suggesting that the site of ATP synthesis resides on the β subunit (Carlier et al., 1979) supports the presence of three catalytic sites per CF_1 (Moroney et al., 1983). Substrate binding at the three active sites is believed to be strongly coupled during the catalytic cycle (Boyer, 1980).

Catalysis appears to involve an S_N2 mechanism (Webb, 1980), and the active conformation of the substrate is known to be a Λ-bidentate nucleotide complex with a divalent metal cation (Frasch & Selman, 1981). The active site also dem-

† This work was supported by grants from the U.S. Department of Agriculture (83-CRCR-1-1339) to W.D.F. and R.R.S. and from the Rackham Foundation and the Phoenix Memorial Research Foundation to W.D.F.

‡ Department of Cellular and Molecular Biology.
§ Department of Chemistry.

[1] Abbreviations: AMPPNP, 5′-adenylyl β-imidodiphosphate; SE, spin-echo; SED, spin-echo difference; CF_1, chloroplast coupling factor 1; DTT, dithiothreitol; WRK, Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate); DCCD, dicyclohexylcarbodiimide.

0006-2960/85/0424-5454$01.50/0