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Reductive Methylation and *p*Ka Determination of the Lysine Side Chains in Calbindin D_{9k}

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The Lys residues in the 75-residue Ca^{2+}-binding protein calbindin D_{9k} were reductively methylated with ^{13}C-enriched formaldehyde. The possible structural effects resulting from the chemical modification were critically investigated by comparing two-dimensional NMR spectra and the exchange rates of some of the amide protons of the native and the modified protein. Our results show that the protein retains its structure even though 10 Lys out of a total of 75 amino acid residues were modified. In the Ca^{2+}- and apo-forms of the protein, the ^{13}C-methylated Lys residues can be detected with high sensitivity and resolution using two-dimensional (^1H, ^{13}C)-heteronuclear multiple quantum coherence (HMQC) NMR spectroscopy. The *p*Ka values of the individual Lys residues in Ca^{2+}-calbindin D_{9k} and apo-calbindin D_{9k} were obtained by combining *p*H titration experiments and (^1H, ^{13}C)-HMQC NMR spectroscopy. Each Lys residue in the Ca^{2+}- and apo-forms of calbindin D_{9k} has a unique *p*Ka value. The Lys *p*Ka values in the calcium protein range from 9.3 to 10.9, while those in the apo-protein vary between 9.7 and 10.7. Although apo-calbindin D_{9k} has a very similar structure compared to Ca^{2+}-calbindin D_{9k}, the removal of two Ca^{2+} ions from the protein leads to an increase of the *p*Ka values of the Lys residues.

KEY WORDS: Calbindin D_{9k}; lysine; reductive methylation; *p*Ka; NMR.

1. INTRODUCTION

Calbindin D_{9k} is a small (75 residues) "EF-hand" calcium-binding protein belonging to the calmodulin superfamily. It acts as a Ca^{2+} transporter in a variety of mammalian cells, such as the intestine, kidney, and uterus (for a review, see Christakos *et al.*, 1989). The protein binds two Ca^{2+} ions in a positive cooperative manner with a K_d value of 10^{-7}–10^{-8} M (Linse *et al.*, 1987, 1991). The C-terminal Ca^{2+}-binding site in calbindin D_{9k} follows the typical "EF-hand" motif, and contains a 12-residue loop which provides seven ligands for Ca^{2+}, as is found in most proteins of the calmodulin superfamily. However, the N-terminal Ca^{2+}-binding site is often referred to as a "pseudo-EF-hand" due to the insertion of two residues in the Ca^{2+}-binding loop and its irregular Ca^{2+} coordination (Vogel *et al.*, 1985). Among the 75 amino acid residues of calbindin D_{9k}, a total of 17 residues are negatively charged Asp or Glu, while 10 are positively charged Lys residues (no Arg is found in the protein). The structure of the Ca^{2+}-saturated form of calbindin D_{9k} has been determined by X-ray crystallography (Szebenyi and Moffat, 1986; A. Svensson *et al.*, 1992) and by NMR (Kördel *et al.*, 1993); these studies reveal a pear-shaped protein molecule. Recent structural studies have shown that calbindin D_{9k} essentially retains its three-dimensional structure upon removal of the two Ca^{2+} ions (Skeleton *et al.*, 1990*a*, *b*). The two Ca^{2+} ions are located at one end of the protein, and

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this region of the protein is highly negatively charged; the other end of the protein has a net positive charge due to the high abundance of the Lys residues in this region. Thus, calbindin D_{9k} has a net polarity due to the uneven charge distribution. A number of studies have shown that the negative surface charge in the protein plays a critical role in determining the Ca^{2+}-binding properties of the protein. Neutralization of some of the negative surface charges close to the Ca^{2+}-binding sites by site-specific mutagenesis significantly decreases the Ca^{2+}-binding affinity (Linse *et al.*, 1988; B. Svensson *et al.*, 1990). Kinetic studies of such mutants have shown that the lower Ca^{2+} affinity is largely due to a decrease in the Ca^{2+} association rate to the protein (Martin *et al.*, 1990). The authors proposed that neutralization of the negative surface charges around the Ca^{2+}-binding sites decreases the protein's electrostatic attraction toward positively charged Ca^{2+} ions (Martin *et al.*, 1990). The electric field gradient resulting from the uneven charge distribution in the protein may also be an important factor in guiding the Ca^{2+} ions to their binding sites and, in addition, could facilitate fast Ca^{2+} association rates. Indeed, a similar electric field gradient has been found to be important in guiding the superoxide anion, O_2^-, to the active site of superoxide dismutase, accounting for the very rapid association rate (even faster than the diffusion rate) of O_2^- to the active site of the enzyme (Getzoff *et al.*, 1983).

In an attempt to clarify the role of the positively charged Lys residues in calbindin D_{9k}, we have reductively methylated all of the Lys residues using $NaCNBH_3$ and ^{13}C-enriched HCHO. The structural effects which might result from the reductive methylation were studied by various NMR techniques. The *p*Ka values of the individual Lys side chains in Ca^{2+}-calbindin D_{9k} as well as apo-calbindin D_{9k} were determined by recording two-dimensional (^1H, ^{13}C)-heteronuclear multiple quantum coherent (HMQC) NMR spectra of the methylated protein at a range of *p*H values.

2. MATERIALS AND METHODS

All chemicals used in this work were analytical grade unless otherwise stated. Carbon-13-enriched formaldehyde, $^{13}CH_3OH$, KOD, DCl, and D_2O were purchased from MSD Isotopes (Montreal, Canada). Bovine calbindin D_{9k} was expressed in and purified from a bacterial expression system as described previously (Brodin and Grundström, 1986; Brodin *et al.*, 1989). The apo-protein was prepared by passing calbindin D_{9k} through a Chelex-100 column equilibrated with 100 mM NH_4HCO_3, *p*H 8.0.

Chemical modification of calbindin D_{9k}: The reductive methylation of calbindin D_{9k} using ^{13}C-enriched (99%) formaldehyde was performed essentially in the same manner as described previously for calmodulin (Huque and Vogel, 1993; Zhang and Vogel, 1993). Briefly, 5 mg calbindin D_{9k} was dissolved in 3 ml of 50 mM HEPES buffer, 10 mM $CaCl_2$, *p*H 7.5. Carbon-13-labeled formaldehyde and freshly prepared $NaCNBH_3$ (1 M solution) were added to this solution in fivefold excess over the free amino groups in the protein, and the mixture was mixed gently by shaking and incubated overnight at 4°C. The reaction was stopped by extensive dialysis against 10 mM NH_4HCO_3, and the solution was subsequently freeze-dried and stored at −20°C prior to the NMR studies.

NMR spectroscopy: All the NMR spectra were acquired at 25°C on a Bruker AMX500 spectrometer using a 5-mm inverse-detection broadband probe. The solvent D_2O was used to provide a spectrometer frequency lock in all experiments. The data were processed using Bruker UXNMR software on an X32 computer. In all two-dimensional (2D) experiments, the data were zero-filled once, and a 72°-shifted-sine-squared window function was applied in both dimensions prior to the Fourier transformation.

DQF-COSY spectra of native and methylated calbindin D_{9k} in H_2O/D_2O (90%/10% by volume) (*p*H 6.5) were recorded using a standard pulse sequence (Rance *et al.*, 1983). The protein concentration of both samples was ≈2.5 mM. A total of 512 experiments with 32 scans per experiment were collected for each spectrum. A sweep width of 6500 Hz with 2048 data points was covered in the F2 dimension. The water resonance was suppressed by a selective presaturation pulse during the relaxation delay.

The amide exchange rates of the slowly exchanging amide protons of Ca^{2+}-calbindin D_{9k} were measured by a direct exchange-out experiment. A concentrated calbindin D_{9k} sample with a concentration of ≈10 mM, *p*H 7.5 in H_2O was diluted tenfold with D_2O at time zero. One-dimensional ^1H NMR spectra of the diluted sample were recorded at ten different time points ranging

from 8 min to 4 days. The intensities of some of the resolved amide protons at different time points after the dilution were measured to derive their exchange rates. The exchange rates for some of the fast-exchanging amides were measured using the saturation transfer technique in the same manner as described by Linse *et al.* (1990) for the same protein.

The *p*H titration experiments of methylated Ca^{2+}-calbindin D_{9k} and apo-calbindin D_{9k} were carried out in D_2O solution using an ≈1.0 mM protein sample in 0.1 M KCl. A small amount of $^{13}CH_3OH$ (1 mM final concentration) was added as an internal chemical shift reference. The titration covers a *p*H range of 2.5–11.5. The *p*H values were direct readings from the *p*H meter; no correction for the solvent isotope effect was applied. A (^1H, ^{13}C)-HMQC spectrum was acquired at each *p*H titration point using the pulse sequence described by Bax *et al.* (1983) in the phase-sensitive mode which was obtained by using the time-proportional phase increment (TPPI) technique (Marion and Wüthrich, 1983). Typically, a 20-ppm spectral width was covered in the ^{13}C dimension with the carrier centered between the frequency of the methyl groups of monomethyllysine and dimethyllysine. The ^1H carrier was set at 3 ppm and the sweep width was 2500 Hz. A total of 160 experiments with eight scans per experiment were collected for each spectrum.

3. RESULTS

One-dimensional ^{13}C NMR spectroscopy was used to determine the extent of methylation. Figure 1 shows the ^{13}C NMR spectrum of methylated Ca^{2+}-calbindin D_{9k}. As shown earlier (Jentoft *et al.*, 1981; Gerken *et al.*, 1982; Huque and Vogel, 1993; Zhang and Vogel, 1993), the resonances in Fig. 1A arise from the dimethyllysine residues and the dimethylated N-terminal amino group. The resonances between 32 and 34 ppm belong to the monomethyl groups that result from incomplete reductive methylation (Fig. 1B). The resonance at ≈42 ppm in Fig. 1A could be assigned to the N-terminal dimethylated amino group because of its low *p*Ka value (*p*Ka ≈ 7.2) and upfield shifting ^{13}C resonance with increasing *p*H. Because of its low *p*Ka the N-terminal -NH_2 group was completely dimethylated; hence, no resonance for the monomethylated N-terminal amine was detected in Fig. 1B. The integration of the resonances

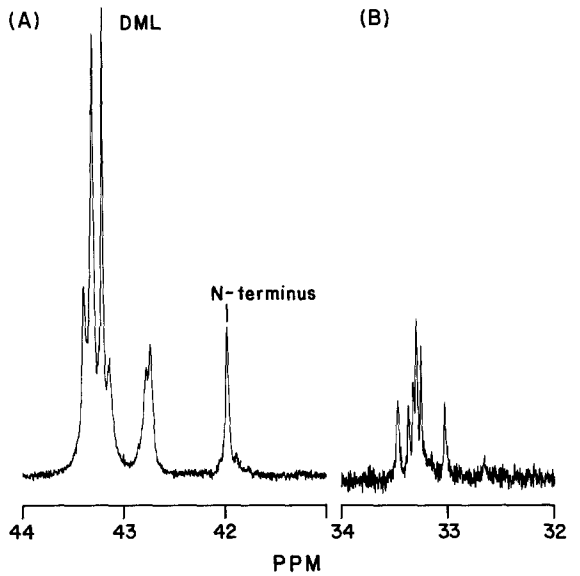

Fig. 1. The 125.8-MHz 1D ^{13}C spectra of reductive methylated Ca^{2+}-calbindin D_{9k}. (A) Resonances arising from the dimethyllysine residues and the dimethylated -NH_2 group; (B) the resonances for the monomethyllysine residues. The spectrum was recorded at 25°C on a 1.0 mM Ca^{2+}-calbindin sample dissolved in 99.9% D_2O containing 0.1 M KCl, *p*H 8.5.

in Fig. 1A and B, respectively, indicates that about 90% of the Lys residues in the protein were dimethylated, and only 10% were monomethylated under the modification conditions used. However, the ratio of dimethylation to monomethylation can be increased further by changing the amount of $^{13}CH_3OH$ and $NaCNBH_3$ used in the reaction and repeating the reaction (data not shown) (Huque and Vogel, 1993). Figure 1 also shows that the resonances in both dimethyl and monomethyl regions do not have the same intensity, suggesting that each Lys residue in the protein has a different reactivity toward the methylation reaction due to the differences in their *p*Ka values (Zhang and Vogel, 1993). Indeed, each Lys residue in the protein does have its own unique *p*Ka value (see below).

Because calbindin D_{9k} has such a high number of Lys residues (10 out of a total of 75 amino acid residues), it is important to investigate whether structural perturbations would result from the complete modification of these residues. Consequently, we have recorded DQF-COSY spectra of native and methylated Ca^{2+}-calbindin D_{9k} under identical experimental conditions. The resonances of native Ca^{2+}-calbindin D_{9k} have been completely

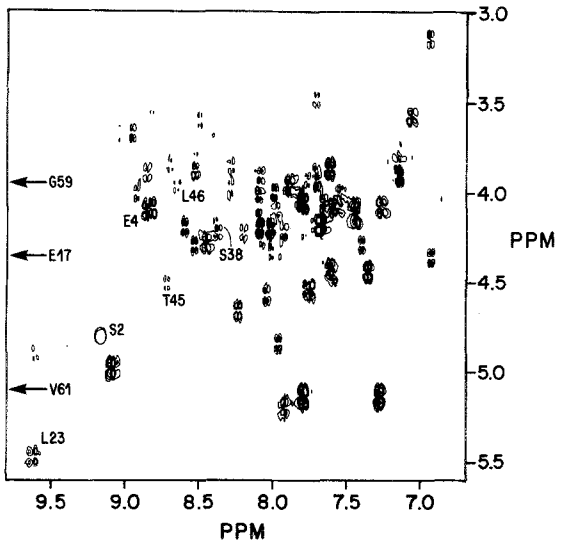

Fig. 2. The fingerprint region of the DQF-COSY spectrum of reductive methylated Ca^{2+}-calbindin D_{9k} (pH 6.5). The resonances in the spectrum can be directly identified by comparing to the spectrum of the native protein (Kördel *et al.*, 1989). The latter was assigned at pH 6.0, and the slight difference in pH in our study caused some of the peaks to be of lower intensity due to the somewhat faster amide exchange rates at pH 6.5. Only the cross-peaks for which the amide exchange rates were measured are labeled.

Table I. Comparison of Some of the NH Exchange Rates of Calbindin D_{9k} Before and After Chemical Modification

Residue	I_{sat}/I_{unsat} [a]	
	Unmodified	Modified
S2	0.63	0.66
E4	0.90	0.93
L23	0.66	0.80
S38	$\log k \geq 0.7$ [b]	$\log k \geq 0.7$ [b]
T45	0.91	0.91
L46	$\log k \geq 0.7$ [b]	$\log k \geq 0.7$ [b]
E17	$\log k = -3.3$ [c]	$\log k = -3.6$ [c]
G59	$\log k = -3.4$ [c]	$\log k = -3.3$ [c]
V61	$\log k = -5.5$ [c]	$\log k = -5.8$ [c]

[a] I_{sat}/I_{unsat} stands for the ratio of the peak volumes of a specific amino acid residue measured with and without presaturation on the H_2O resonance.

[b] In these cases, presaturation leads to almost complete disappearance of the peaks in both the unmodified and modified protein samples.

[c] $\log k$ values were derived from exchange-out experiments for the slowly exchanging amides. Spectra were normalized using the nonexchangeable aromatic resonances as an internal standard.

assigned (Kördel *et al.*, 1989) and are used directly here. Figure 2 shows the fingerprint region of the DQF-COSY spectrum of methylated Ca^{2+}-calbindin D_{9k} at pH 6.5, and the spectrum is identical to that of the native protein (see also Linse *et al.*, 1987), suggesting that methylated calbindin D_{9k} retains its three-dimensional structure after the methylation reaction. The possible structural perturbation from the reductive methylation was further studied by measuring the exchange rates for some of the amide protons in native and methylated Ca^{2+}-calbindin D_{9k}. For the fast-exchanging amide protons, the exchange rates were measured by the saturation transfer technique as shown previously (Linse *et al.*, 1990). No attempt was made to measure the exact values of the exchange rates, but instead, the ratio of the resonance intensity with and without presaturation of the water resonance was used to assess whether the dimethylation resulted in any changes in the amide exchange rates. Table I lists the values of I_{sat}/I_{unsat} for some of the fast-exchanging amides before and after reductive methylation. It is clear that these amide protons maintain the same I_{sat}/I_{unsat} values following the reductive methylation, indicating that these amide protons have the same exchange rates as found in the native protein. The exchange rates of some of the slow-exchanging amide protons were measured by direct exchange-out experiments. The exchange rates k were extracted by least-square fitting of the peak intensity to the equation $I = I_0 + A\exp(-kt)$. The measured amide exchanges are reported in terms of $\log k$ in Table I. Again, the exchange rates for these slowly exchanging NHs remain the same within experimental error after chemical modification (see Table I). We also found that the UV spectra of native and modified calbindin were identical (data not shown). Hence, the results strongly support the notion that the methylation of the Lys residues does not result in observable structural or dynamical changes in calbindin D_{9k}.

It has been shown earlier that pH titration experiments of methylated proteins together with one-dimensional (1D) ^{13}C NMR can serve to obtain pKa values of the Lys residues in some proteins (Jentoft *et al.*, 1981; Gerken *et al.*, 1982; Huque and Vogel, 1993). However, this experimental

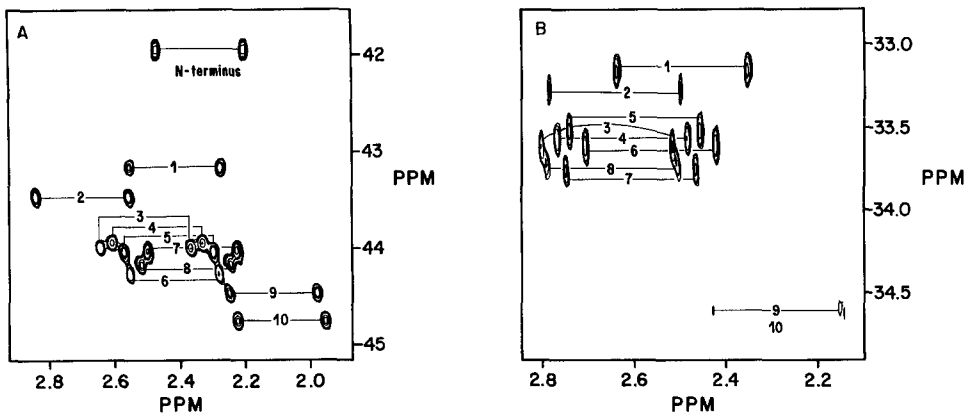

Fig. 3. The (^{1}H, ^{13}C)-HMQC spectrum of reductive methylated Ca^{2+}-calbindin D_{9k} recorded at 25°C, *p*H 10.0. No decoupling was applied during the acquisition; hence, each methyl resonance appears as a doublet. (A) Dimethyllysine region; (B) monomethyllysine region. The chemical shifts were calibrated referring to the methyl group of internal $^{13}CH_3OH$ at 49.5 ppm and 3.36 ppm for ^{13}C and ^{1}H, respectively.

approach suffers from poor spectral resolution during the *p*H titrations. Fortunately, as we have shown earlier (Zhang and Vogel, 1993; Zhang *et al.*, 1994), the 2D (^{1}H, ^{13}C)-HMQC NMR spectroscopic technique provides excellent resolution as well as sensitivity in detecting both dimethyl and monomethyl groups of Lys residues in proteins. Figure 3 shows the dimethyllysine and monomethyllysine regions of the (^{1}H, ^{13}C)-HMQC spectrum of methylated Ca^{2+}-calbindin D_{9k} at *p*H 10.0. No ^{13}C decoupling was applied during the data acquisition time; hence, each methyl resonance appears as a doublet. The two methyl groups from a dimethyllysine are degenerate due to the fast rotation of the C_ε–N bond; thus, only one ^{13}C chemical shift is measured for each dimethyllysine. It can be seen that it is possible to resolve all ten Lys residues in the dimethyllysine region, whereas nine residues are seen in the monomethyllysine region of the spectrum (Fig. 3A and B). The unique chemical shift values for each dimethyllysine and monomethyllsine residue indicate that each Lys residue in the protein is in a unique microenvironment. The well-resolved 2D (^{1}H, ^{13}C)-HMQC spectra allowed us to determine the *p*Ka values for each Lys residue in Ca^{2+}-calbindin D_{9k}. All the resonances in Fig. 3 show monophasic titration curves; the absence of biphasic curves indicates the absence of strong salt linkages (Gerken *et al.*, 1982; Huque and Vogel, 1993). Figure 4 gives an example of the *p*H titration curves obtained for resonances 5 and 9 in Fig. 3A. The *p*Ka values for each dimethyllysine and monomethyllysine resonance were derived by nonlinear least square fitting of the titration curves using the Simplex algorithm (Caceci and Cacheris, 1984); and the values obtained for Ca^{2+}-calbindin

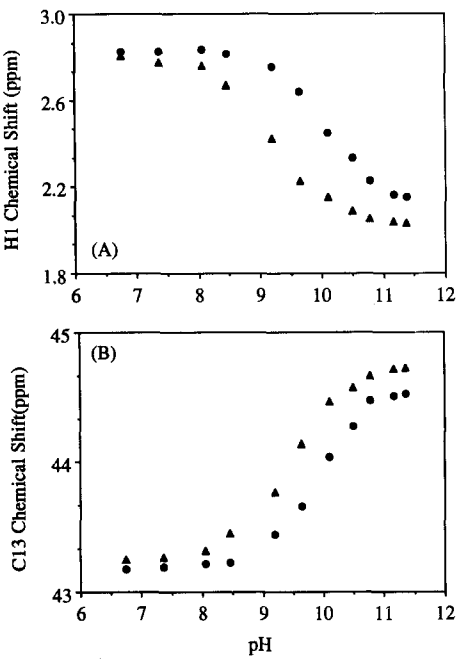

Fig. 4. The *p*H titration curves for the dimethyl peaks 5 and 9 in Fig. 3A. The top and bottom panels represent the titration curves derived from the ^{1}H and ^{13}C chemical shifts, respectively. The ^{1}H resonances shift upfield and the ^{13}C resonances shift downfield with increasing *p*H; this behavior is normal for dimethyl Lys resonances. The monomethyl Lys resonances behave in the same way (data not shown).

Table II. pKa Values of the Lysine Residues in Ca^{2+}-Calbindin D_{9k}

	pKa of dimethyllysine			pKa of monomethyllysine		
Resonance	From ^1H	From ^{13}C	Mean	From ^1H	From ^{13}C	Mean
1	10.57	10.56	10.57	11.06	11.10	11.08
2	10.90	10.90	10.90	11.35	11.31	11.33
3	10.14	10.08	10.11	10.95	11.05	11.00
4	10.10	10.07	10.09	10.81	10.91	10.86
5	10.06	9.92	9.99	10.90	10.91	10.91
6	9.99	9.77	9.88	10.74	10.77	10.76
7	9.92	9.87	9.90	10.66	10.66	10.66
8	9.99	9.79	9.89	10.86	10.87	10.87
9	9.25	9.48	9.37	10.18	10.15	10.17
10	9.35	9.32	9.34	10.18	10.15	10.17
$-NH_2$[a]	7.25	7.13	7.19			

[a] The free amino group at the N-terminus of the protein.

D_{9k} are listed in Table II. In agreement with the different chemical shift values observed in Fig. 3, each Lys residue in the protein has a unique pKa value, which reinforces the notion that each Lys residue is in a unique local environment.

We have also studied methylated apo-calbindin D_{9k} in the same fashion. Figure 5 shows a 2D (^1H, ^{13}C)-HMQC spectrum of the methylated apo-protein at pH 10.0. Similar to what is shown in Fig. 3, the resonances in the dimethyllysine region as well as the monomethyllsine region are well resolved. However, the removal of two Ca^{2+} ions from the protein induces significant spectral changes, as revealed by comparing Figs. 3 and 5, suggesting that the pKa values of the Lys residues also change upon the removal of Ca^{2+} ions. Indeed, the pKa values for the Lys residues in apo-calbindin D_{9k}, which were obtained for monophasic pH titration curves in the same manner as described for the Ca^{2+} form of the protein, show significant differences compared to those in the Ca^{2+} form of the protein.

During the pH titration of apo-calbindin D_{9k}, we observed severe line broadening, leading to an undetectable signal for the dimethyl resonance of the N-terminal $-NH_2$ group around its pKa value (pH 6–8). This behavior generally arises when the diastereotopic methyl groups are in two different conformations which are slowly exchanging. Alternatively, the two slowly exchanging conformers could be the protonated form which may be involved in a salt linkage with a negatively charged group in the protein, and the deprotonated form which is no longer involved in this ion pair.

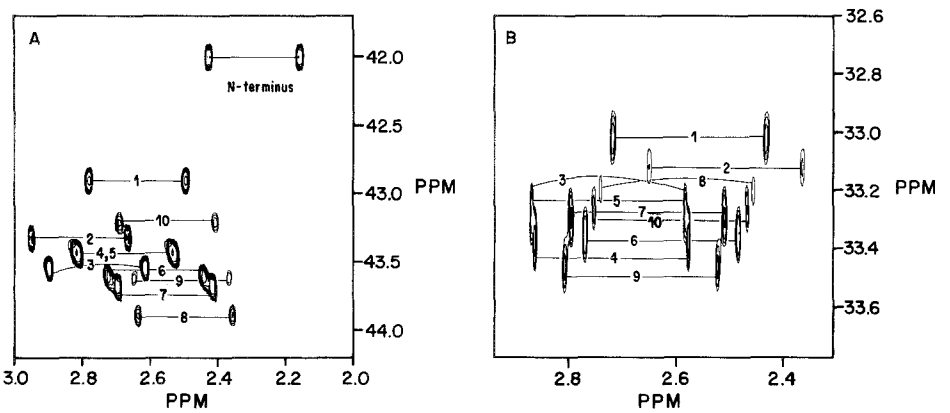

Fig. 5. The (^1H, ^{13}C)-HMQC spectrum of reductive methylated apo-calbindin D_{9k}. The spectrum was recorded under the same conditions as described in Fig. 3. (A) Dimethyllysine region; (B) monomethyllysine region.

4. DISCUSSION

Determination of the *p*Ka values of Lys residues in proteins by experimental and theoretical approaches represents an active field of research (Jentoft and Dearborn, 1983; Bashford and Karplus, 1990; Yang *et al.*, 1993; Zhang and Vogel, 1993). However, most of the currently available experimental approaches are either not very reliable or are time-consuming (Lundblad and Noyes, 1984; Fersht, 1985). To date, the theoretical methods have had only modest success in predicting accurate *p*Ka values for titratable groups in proteins; this is largely due to the oversimplification in the theoretical models and the very limited range of reliable experimental data to refine the current models (Bashford and Karplus, 1990; Yang *et al.*, 1993). We earlier presented an experimental approach which combines a reductive methylation reaction and 2D(^{1}H, ^{13}C)-HMQC NMR spectroscopy to derive reliable *p*Ka values for individual Lys side chains in proteins using calmodulin as a model system (Zhang and Vogel, 1993; Zhang *et al.*, 1994). Here, we have extended this experimental approach to another protein, calbindin D_{9k}, in which Lys residues are highly abundant and may be involved in guiding the Ca^{2+} ions to their proper binding sites. The reductive methylation reaction was employed to introduce one or two -$^{13}CH_3$ group(s) on the ε-NH_2 groups of the Lys residues and α-NH_2 group of the N-terminus in calbindin D_{9k} (Jentoft and Dearborn, 1983; Huque and Vogel, 1993). Unlike most other chemically modified Lys residues (Lundblad and Noyes, 1984), both monomethylated and dimethylated Lys residues retain a positive charge and are titratable; these characteristics are the most important features of a Lys residue. Theoretical calculations have shown that monomethylation and dimethylation have only a small effect on the capacity of a Lys residue to participate in ion pair formation (Mavri and Vogel, 1994). The dimethylated Lys residue has a *p*Ka value which is very close to that of an unmodified Lys; while the monomethylated Lys has a better hydrogen-bonding capacity, but it suffers from a slightly higher *p*Ka value compared to Lys. Be that as it may, the *p*Ka differences are less than 1 *p*H unit (Jentoft and Dearborn, 1983; Huque and Vogel, 1993). Thus, the methylation reaction should introduce minimal perturbations to the function of a protein. Indeed, it has been shown earlier that the complete methylation of the Lys residues in a related Ca^{2+}-binding protein, calmodulin, does not affect its ability to activate its target enzyme, cyclic nucleotide phosphodiesterase (Huque and Vogel, 1993). Furthermore, recent studies have shown that the crystal structures of native and methylated lysozyme are identical (Rypiewsky *et al.*, 1993).

Both the fingerprint region of the DQF-COSY spectrum (Fig. 2) of the methylated protein and the exchange rates of some of the amide protons (Table I) clearly show that calbindin D_{9k} retains its three-dimensional structure as well as its dynamics after 10 Lys residues out of a total of 75 amino acid residues are modified. These results ensure that the *p*Ka values of the Lys residues derived from the modified protein reflect their true values. The excellent resolution and sensitivity of the 2D (^{1}H, ^{13}C)-HMQC spectra shown in Figs. 3 and 5 allowed us to resolve the individual Lys resonances for both Ca^{2+}-calbindin and apo-calbindin. Hence it was possible to obtain a reliable *p*Ka value for each Lys residue in the apo and Ca^{2+} forms of the protein by performing *p*H titration studies (Tables II and III). These results show that the experimental approach used in this work has general applicability.

The (^{1}H, ^{13}C)-HMQC spectrum of Ca^{2+}-calbindin D_{9k} is significantly different from that of apo-calbindin D_{9k}. The resonances of apo-calbindin D_{9k} only span an $\approx$1-ppm and 0.5-ppm chemical shift range in the ^{13}C dimension for the dimethyl and monomethyl Lys residues, respectively (Fig. 5). Those from Ca^{2+}-calbindin D_{9k} are spread out over $\approx$1.8 ppm and 1.5 ppm, respectively (Fig. 3). Similar differences in the chemical shift distribution are also observed in the ^{1}H dimension (Figs 3 and 5). The above data indicate that the Lys residues in the apo-protein have quite similar microenvironments. The binding of Ca^{2+} induces local structural changes of some of the Lys residues, and brings them into a more unique environment, although the apo and Ca^{2+} forms of calbindin D_{9k} have a very similar overall 3D structure (Skelton *et al.*, 1990*a*, *b*). The local changes of the Lys residues resulting from the Ca^{2+} binding to the protein are also reflected in the changes of their *p*Ka values. In apo-calbindin, only one Lys residue (peak '1' in Fig. 5) has an unusual *p*Ka value (10.7), and the rest of the Lys residues have *p*Ka values close to that found for the amino acid derivative dimethyllsine (*p*Ka = 10.0) (Huque and Vogel, 1993). On the other hand, four Lys residues in Ca^{2+}-calbindin

Table III. pKa Values of the Lysine Residues in Apo-Calbindin D_{9k}

	pKa of dimethyllysine			pKa of monomethyllysine		
	From ^1H	From ^{13}C	Mean	From ^1H	From ^{13}C	Mean
1	10.71	10.70	10.71	11.31	11.53	11.42
2	10.36	10.46	10.41	11.00	11.30	11.15
3	10.21	10.13	10.17	11.68	11.74	11.71
4	10.13	10.38	10.27	11.20	11.14	11.17
5	10.17	10.29	10.23	11.20	11.38	11.24
6	9.98	10.00	9.99	10.99	11.13	11.06
7	9.97	9.89	9.93	11.10	11.13	11.12
8	9.73	9.75	9.74	10.78	10.84	10.81
9	10.27	10.38	10.33	10.59	10.90	10.75
10	9.87	9.91	9.89	11.10	11.13	11.12
$-NH_2$	ND[a]	ND[a]	ND[a]			

[a] pKa value could not be obtained due to the severe line broadening around pH 8.0.

D_{9k} have unusual pKa values; resonances 1 and 2 have high pKa values of 10.6 and 10.9; and resonances 9 and 10 have significantly lower pKa values of 9.4 and 9.3, respectively. The range of the pKa values of the Lys residues in Ca^{2+}-calbindin D_{9k} is 1.6 pH units; for apocalbindin this number is 1 pH unit. The average pKa values of the dimethyl and monomethyllysine residues in calcium calbindin are 10.0 and 10.8; for apo calbindin these numbers are 10.2 and 11.1. Such differences in the pKa values of monomethyl versus dimethyl resonances are normally observed. Interestingly, the binding of Ca^{2+} to the protein leads to an overall decrease in the Lys pKa values, and this could result from the overall charge neutralization by the two positively charged calcium ions, as well as from local structural changes that occur upon the binding of Ca^{2+} ions. A similar pKa decrease as a result of Ca^{2+} binding was observed for calmodulin, a related acidic protein (Zhang and Vogel, 1993). We have attempted to make the specific assignments of the methyl resonances in Figs. 3 and 5 using various heteronuclear correlation or nuclear Overhauser enhancement based experiments. Due to the small coupling constants and the overlap of the methyl protons with the ε-CH_2 protons of the Lys side chains, these attempts were unsuccessful. In studies of calmodulin, we used site-directed mutagenesis of all Lys residues to obtain this assignment (Zhang and Vogel, 1993); however, such mutants are not available for calbindin. Thus, we cannot assign the dramatic changes in the pKa values resulting from the binding of Ca^{2+} to the protein to any specific Lys residues. All the Lys residues in Ca^{2+}-calbindin D_{9k} are located on the surface of the protein in the X-ray structure of the protein (Szebenyi and Moffat, 1986; A. Svensson *et al.*, 1992; Kördel *et al.*, 1993). The low pKa values observed for resonances 9 and 10 may indicate that they are located in a positively charged environment. Furthermore, the resonances 1 and 2 may be in a more negatively charged environment; however, it should be noted that no salt linkages, which will lead to higher pKa values for Lys residues, were found in the crystal structure of the protein (Szebenyi and Moffat, 1986). The absence of biphasic pH titration curves noted in this work indicates that hydrogen-bonded salt linkages are also absent in solution. The high-resolution structure of Ca^{2+}-calbindin D_{9k} in solution as derived from NMR studies has shown that some of the Lys side chains are conformationally constrained, suggesting that interactions with other regions of the protein take place (Kördel *et al.*, 1993).

In conclusion, our data show that the carbon-13 methylation and HMQC NMR approach provides a relatively straightforward means to determining individual Lys pKa values in proteins. In proteins with a very high charge density, such as calbindin and calmodulin, the modification does not perturb the protein structure and dynamics; moreover, each Lys has a unique pKa value which reflects the electrostatics of its environment.

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