Characterization of pH Titration Shifts for All the Nonlabile Proton Resonances in a Protein by Two-Dimensional NMR: The Case of Mouse Epidermal Growth Factor†

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Received December 27, 1990; Revised Manuscript Received February 28, 1991

ABSTRACT: The pH titration shifts for all the nonlabile proton resonances in a 53-residue protein (mouse epidermal growth factor) were measured in the p^2H range 1.5–9 with two-dimensional (2D) ^1H NMR. The 2D NMR pH titration experiment made it possible to determine the pK values for all the ionizable groups which were titrated in the pH range 1.5–9 in the protein. The pK values of the nine ionizable groups (α-amino group, four Asp, two Glu, one His, and α-carboxyl group) were found to be near their normal values. The 2D titration experiment also provided a detailed description of the pH-dependent behavior of the proton chemical shifts and enabled us to characterize the pH-dependent changes of protein conformation. Analysis of the pH-dependent shifts of ca. 200 proton resonances offered evidence of conformational changes in slightly basic pH solution: The deprotonation of the N-terminal α-amino group induced a widespread conformational change over the β-sheet structure in the protein, while the effects of deprotonation of the His22 imidazole group were relatively localized. We found that the 2D NMR pH titration experiment is a powerful tool for investigating the structural and dynamic properties of proteins.

Among the early NMR studies of proteins, the pH titration experiment using one-dimensional (1D)[1] spectra produced many interesting results (Jardetzky & Roberts, 1981). The overlaps of proton resonances in 1D NMR spectra, however, limited the available amount of information, and it seems to be out of date for current NMR spectroscopists. By use of two-dimensional (2D) NMR spectra, the chemical shifts of almost all of the proton resonances in proteins with molecular weights up to 10K can be readily identified from isolated cross-peaks. Obviously, the advantage of the pH titration experiment with 2D NMR spectra can provide detailed electrostatic properties of the proteins using all protons as probes which are distributed over a protein globule. However, very few such experiments have been reported since it demands large amounts of time and work. It is to be noted here that the pH titration in the fingerprint region of 2D COSY (correlation spectroscopy) spectra (pH 3–6) has been used to determine the amide proton titration shifts in bull seminal inhibitor IIA (Ebina & Wüthrich, 1984).

Mouse epidermal growth factor (EGF) is a small peptide hormone with a molecular weight of 6K which promotes cell proliferation through a mechanism involving the binding of EGF to specific receptors on the surface of target cells (Carpenter & Cohen, 1979). The sequence of the 53 amino acid residues is known (Savage et al., 1972), and the secondary and tertiary structures were determined by NMR (Montelione et al., 1986, 1987; Kohda et al., 1988a). Mouse EGF consists of two structural domains: the N-terminal domain (1–32) and the C-terminal domain (33–53). Each domain contains a β-sheet structure. In this study, we recorded 12 2D HOHAHA (homonuclear Hartmann–Hahn spectroscopy) spectra of mouse EGF in ^2H_2O at different p^2H values between 1.5 and 9 and analyzed the pH titration curves for ca. 200 nonlabile proton resonances. We found that the 2D NMR pH titration experiment contains much valuable information on the protein structure and dynamics, which otherwise cannot be obtained.

Materials and Methods

Materials. EGF purified from submaxillary glands of adult male mice by chromatography on Bio-Gel P10 and DEAE-cellulose (Savage & Cohen, 1972) was further purified by reversed-phase HPLC (Kohda & Inagaki, 1988).

NMR Measurements. 400-MHz ^1H NMR spectra were recorded on a JEOL JNM-GX400 spectrometer at a probe temperature of 28 °C. The p^2H values given were direct pH meter readings measured at 25 °C with a Radiometer PHM84. The p^2H of the sample solution was adjusted by addition of ^2HCl or NaO^2H. No extra salt was added to the sample. On the addition of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) to the sample as an internal standard, the DSS methyl peak broadened and split, indicating DSS weakly interacted with mouse EGF. Thus, chemical shifts were read from a sharp impurity peak at 0.152 ppm. This posed no problems: Only a minute systematic shift (<0.02 ppm) occurred during the pH titration.

Mouse EGF sample was dissolved at 3.3 mM in 99.95% ^2H_2O in a standard 5-mm tube. The p^2H value was about 2.8 when the lyophilized EGF powder was dissolved in ^2H_2O. HOHAHA spectra (Bax & Davies, 1985) (τ_{mix} = 45 ms) were recorded with 256 × 1024 data points at different p^2H values in the phase-sensitive mode (States et al., 1982). First the p^2H of the sample was adjusted to 1.59, then 2.70, 3.30, 3.70, 4.16, 4.88, 5.29, 6.38, 6.89, 7.71, 8.30, and 8.79, and finally back to 7.26 to ensure the reversibility of the titration. The sample

†This work was supported by grants from the Human Frontier Science Program Organization and the Ministry of Education, Science and Culture of Japan.

[1] Abbreviations: 1D, one dimensional; 2D, two dimensional; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EGF, epidermal growth factor; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; NOE, nuclear Overhauser enhancement.

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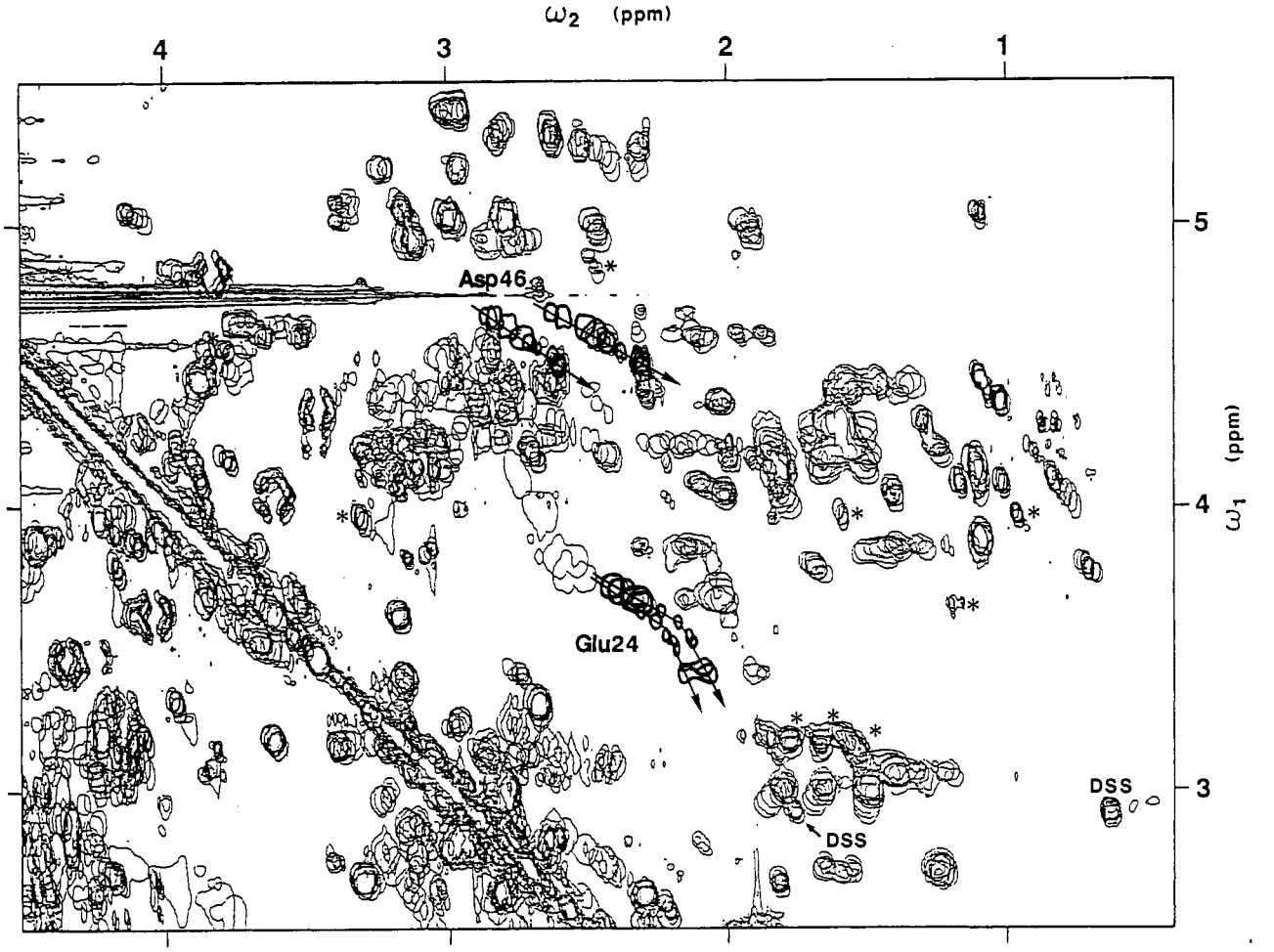

FIGURE 1: Overlaid plot of the $C^{\alpha}H(\omega_1)$–aliphatic (ω_2) regions of 12 HOHAHA spectra recorded at 28 °C in ^2H_2O in the range from p^2H 1.6 to 8.8. The cross-peaks from 2,2-dimethyl-2-silapentane-5-sulfonate are marked with "DSS". The cross-peaks from impurities (including the methionine sulfoxide derivative of mouse EGF) or a minor conformer are marked with asterisks.

solution became turbid in the p^2H range from 3.5 to 6.5, but no special care was taken. Since EGF was precipitated near the isoelectric point, p*I* 4.6 (Matrisian et al., 1984), the HOHAHA spectrum at p^2H 4.88 was not used due to the low signal-to-noise ratio. A total of 128–256 scans were accumulated for each t_1 with a spectral width of 5000 Hz. The digital resolution was 9.8 Hz or 0.02 ppm/point in both dimensions by zero-filling in the t_1 dimension. A phase-shifted sine-bell function was applied for both t_1 and t_2 dimensions. After Fourier transformation, t_1 noise was reduced by the subtraction method (Klevit, 1985).

Analysis of pH Titration Curves. The apparent p*K*s (pK_{app}) of titrated proton resonances were obtained by nonlinear least-squares fits of the chemical shifts at various p^2H values to a theoretical titration curve (Shrager et al., 1972). The ionization constant, p*K*, of an ionizable group was determined as an average of the pK_{app} values of the protons on the same residue nearest to the ionizable group. The proton resonances showing pK_{app} with a standard deviation less than or equal to 0.2 were considered as affected by the deprotonation of the ionizable group having the closest p*K* value. The resonances of which chemical shift changes were small (typically less than 0.05 ppm), and hence pK_{app}s determined with standard deviations more than 0.2 were regarded as pH-independent.

Results

Figure 1 shows an overlaid plot of 12 2D HOHAHA spectra recorded in ^2H_2O at different p^2H values. The chemical shifts at different p^2H values of the aromatic proton resonances have been obtained from the 1D NMR pH titration experiment in the previous study (Kohda et al., 1988b). In the present study, the pH-dependent chemical shifts of the aliphatic proton resonances were obtained from the 2D HOHAHA spectra measured at different p^2H values. Since the sequential assignment at pH 2.0 has already been performed (Kohda & Inagaki, 1988), the sequence-specific resonance assignments at arbitrary pH values can be obtained by following the titration shifts of the cross-peaks starting from those at pH 2.0. Thus, we have obtained the pH-dependent chemical shifts of the 228 assigned nonlabile proton resonances in mouse EGF in the p^2H range 1.5–9.

In Figure 1, the cross-peaks $C^{\alpha}H,C^{\beta}H$ and $C^{\alpha}H,C^{\beta'}H$ of Asp46 and $C^{\alpha}H,C^{\gamma}H$ and $C^{\alpha}H,C^{\gamma'}H$ of Glu24 are drawn in thick lines. The titration behavior of those cross-peaks from acidic pH to basic pH is indicated with arrows. For the Asp46 residue, $C^{\beta}H,C^{\beta'}H$ (Figure 2G) and also $C^{\alpha}H$ showed titration shifts in the acidic range with the p*K* of its own side-chain carboxyl group. Hence, the cross-peaks $C^{\alpha}H,C^{\beta}H$ and $C^{\alpha}H,C^{\beta'}H$ of Asp46 moved along straight lines as a function of pH (Figure 1). For the Glu24 residue, on the other hand, $C^{\gamma}H,C^{\gamma'}H$ (Figure 2D) and $C^{\alpha}H$ showed biphasic titration shifts in the acidic range with the p*K* of its side-chain carboxyl group and in the neutral range with the p*K* of the N-terminal α-amino group and/or the imidazole group of His22. Thus, the cross-peaks $C^{\alpha}H,C^{\gamma}H$ and $C^{\alpha}H,C^{\gamma'}H$ of Glu24 moved along lines with a kink. Generally, a cross-peak moves along a straight line if both proton resonances on the ω_1 axis and on the ω_2 axis are affected by the same ionizable group.

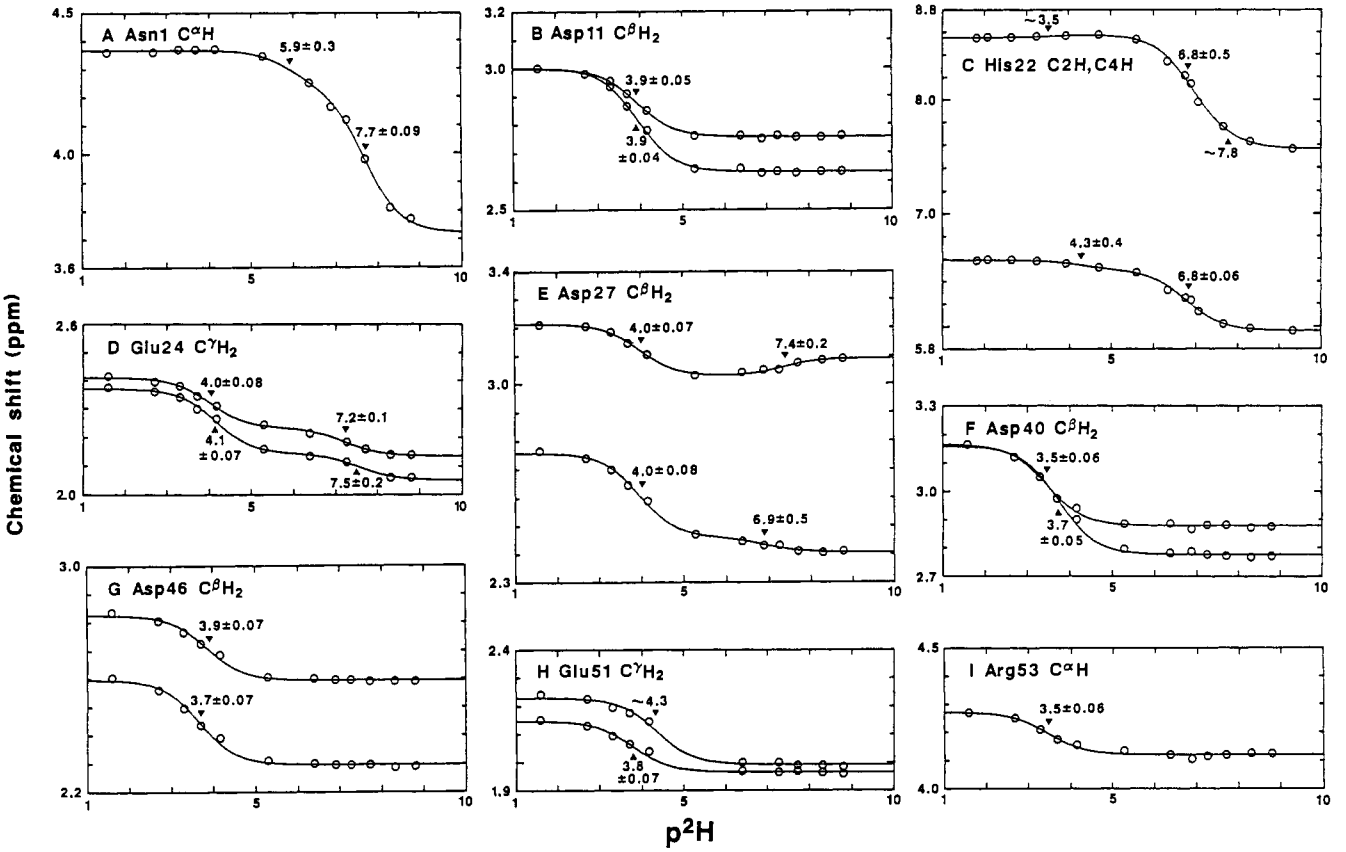

FIGURE 2: p^2H dependence of the chemical shifts of proton resonances used to determine the pKs of the ionizable groups which titrate in the pH range from 1.5 to 9. The chemical shifts were read from the 2D HOHAHA spectra (Figure 1), except for His22 C2H and C4H resonances whose chemical shifts were read from 1D NMR spectra. Some chemical shifts were unavailable due to overlaps of cross-peaks. The fitted titration curves and calculated pK_{app} values with standard deviations are shown.

Figure 2 shows the p^2H dependence of the chemical shifts of several proton resonances used to determine the ionization constants of the ionizable groups in mouse EGF. Titration shifts were analyzed by nonlinear least-squares fits, and the obtained pKs are listed in Table I. The determined pK values are all near their normal values, implying that these ionizable groups form neither salt bridges nor hydrogen bonds.

Detailed analysis of the titration shifts will yield information to characterize the structural and electrostatic properties of mouse EGF in solution. The titration curves of the 95 proton resonances with titration shifts larger than 0.05 ppm were fitted to theoretical curves by nonlinear least-squares fits (data not shown). Then we investigated the effects of deprotonation of the His22 imidazole group, and of the N-terminal α-amino group. Since these pK values differed by only 0.9 pH unit (Table I), the effects of these two groups were difficult to be separated from each other, and hence classified into three types according to their apparent pK values. In the case of $6.6 \leq pK_{app} \leq 7.0$, the proton resonance was designated "H"-type because it was affected only by the deprotonation of the His22 imidazole (pK = 6.8). Note that the pK_{app} range was chosen so that the pK of His22 was always within the error range (± standard deviation) of each pK_{app}. In the case of $7.5 \leq pK_{app} \leq 7.9$, the "N"-type, it was only affected by the N-terminal α-amino group (pK = 7.7). In the case of $7.0 < pK_{app} < 7.5$, the "m(ixed)"-type, the proton resonance was affected by both groups. The amino acid residues containing the affected proton resonances are mapped onto the secondary structure of the N-terminal domain of mouse EGF (Figure 3). The effect of deprotonation of the His22 imidazole group was local on the residues near the imidazole ring: All infuenced protons were within 7 Å from the His22 ring. This indicates that the main effect of deprotonation of His22 is electrostatic and only a slight conformational transition was accompanied by deprotonation of the imidazole group. In contrast, the effect of deprotonation of the N-terminal α-amino group spread widely over the residues on the β-sheet in the N-terminal domain: The most distant proton was 15 Å away from the N-terminal amino group, suggesting that the deprotonation of the α-amino group induced a conformational change over the N-terminal domain of mouse EGF. Interestingly, the deletion of the N-terminal Asn1 residue also affected the proton resonances of the same

Table I: Ionization Constants of the Ionizable Groups in Mouse Epidermal Growth Factor at 28 °C in ^2H_2O[a]

residue	group	pK	pK_{int}[b]
Asn1	α-NH_3^+	7.7 ± 0.1	7.7
Asp11	β-COOH	3.9 ± 0.05	4.0
His22	imidazole	6.8 ± 0.1	6.8
Glu24	γ-COOH	4.1 ± 0.1	4.5
Asp27	β-COOH	4.0 ± 0.1	4.0
Asp40	β-COOH	3.6 ± 0.1	4.0
Asp46	β-COOH	3.8 ± 0.1	4.0
Glu51	γ-COOH	~4[c]	4.5
Arg53	α-COOH	3.5 ± 0.1	3.6

[a] In the acidic pH range, the pK values determined in ^2H_2O are approximately 0.06 pH unit higher than the corresponding pK in ^1H_2O. In the basic pH range, the pK values in ^2H_2O are approximately 0.1 unit higher than that in ^1H_2O (Bundi & Wüthrich, 1979). Mouse EGF contains five Tyr residues, but the pK values were not determined because the intrinsic pK value of Tyr was outside of the experimental pH range. Mouse EGF contains no Lys residues. [b] Intrinsic pK value, the expected value it would have in the absence of any influence from the other charged sites on the protein (Honig & Hubbell, 1984; Tanford & Kirkwood, 1957). Data from States & Karplus (1987). [c] The two pK_{app} of $C^{\gamma}H$ and $C^{\gamma'}H$ are significantly different for reliable pK determination (see Figure 2H).

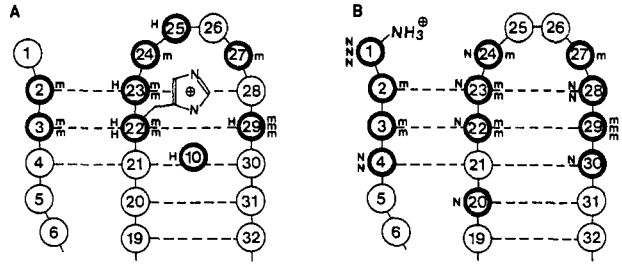

FIGURE 3: Effects of deprotonation of (A) the imidazole group of His22 and (B) the N-terminal α-amino group of Asn1 on the chemical shifts of the proton resonances in mouse EGF. These effects are mapped onto the schematic drawings of the secondary structure of the N-terminal domain in mouse EGF. No effects were seen on the C-terminal domain of mouse EGF. Each symbol, "H", "N", and "m", means a proton resonance that was influenced by the deprotonation of His22, the N-terminal amino group, and both, respectively. See Results for the definition. The residues containing at least one influenced proton resonance are boldfaced.

residues (Kohda et al., 1988b).

Nuclear Overhauser enhancement (NOE), which provides data on internuclear distances, is more directly correlated with the protein conformation than the chemical shift. We compared three 2D NOESY (NOE spectroscopy) spectra recorded at p^{2}H 2.0, 6.8, and 8.7 (τ_{mix} = 80 ms, unpublished results) to investigate the effects of deprotonation of His22 and the N-terminal α-amino group. With the present semiquantitative interpretation of NOEs, however, we failed to detect the conformational change that had been shown by the chemical shift changes. On the other hand, the existence of conformational changes in a near-neutral pH solution was confirmed by the pH titration experiment with circular dichroic spectra (unpublished results). Thus, chemical shifts are a more sensitive parameter for detection of conformational changes than NOEs (Folkers et al., 1989).

The deprotonation of the seven carboxyl groups in mouse EGF induced minor conformational changes, which were shown by means of circular dichroic spectra (unpublished results). The NMR analysis of these conformational changes was difficult, however, because the effects of these carboxyl groups were overlapped in space, and could not be separated.

Discussion

We measured 12 2D HOHAHA spectra of mouse EGF in ^2H_2O in the p^{2}H range 1.5–9 (Figure 1). The total measuring time was about 1 month. Since the chemical shift of a certain resonance can be read from one well-resolved cross-peak among several related ones, the chemical shift change of the specific proton resonance can be easily followed. This is a great advantage of the 2D pH titration study compared with the 1D version. Among the 228 nonlabile proton resonances in mouse EGF, the pH titration behavior of 209 proton resonances could be identified unambiguously by using well-resolved cross-peaks of the 12 2D HOHAHA spectra. The cross-peak could be easily followed when we kept in mind the fact that it tends to move along a straight line or kinked line during pH titration (Figure 1). The accuracy of the chemical shifts in the 2D experiment is ±0.02 ppm in comparison with ±0.001 ppm in the 1D experiment. The titration curves for 95 proton resonances were fitted to theoretical curves, and the pK_{app}s were calculated. It took about a total of 2 weeks to analyze the experimental data. The 2D NMR pH titration experiment offers valuable information on proteins as follows:

Sequence-Specific Resonance Assignments at Arbitrary pHs. Complete sequence-specific resonance assignment of NMR spectra is a prerequisite for NMR to exert its potential power. We can get the resonance assignments by tracing the cross-peaks on a series of 2D NMR spectra starting from a pH value where the sequential assignment has already been completed. Thus, we obtain the sequence-specific resonance assignments of protons at arbitrary pH values within the experimental pH range even above pH 7, where the sequential assignment procedure (Wüthrich, 1986) is usually difficult due to the rapid exchange of amide protons with solvent.

pK Values of All the Ionizable Groups. The ionization constant, pK, of an ionizable group is determined by analyzing the pH titration curves of the protons nearest to the ionizable group on the same residue. For example, C$^\alpha$H is used for the determination of the pK value of terminal α-amino or α-carboxyl groups, C$^\beta$H$_2$ for the β-carboxyl group of Asp, C$^\gamma$H$_2$ for the γ-carboxyl group of Glu, C$^{\epsilon 1}$H (C2H) and C$^{\delta 2}$H (C4H) for the imidazole group of His, C$^\epsilon$H$_2$ for the ϵ-amino group of Lys, and C$^{\epsilon 1}$H and C$^{\epsilon 2}$H (C3,5H) for the ζ-hydroxyl group of Tyr. In the case of mouse EGF, the pK values of the nine ionizable groups were determined with the standard deviation less than 0.1 except for Glu51 (Figure 2 and Table I). All the pK values (α-amino group, four Asp, two Glu, one His, and α-carboxyl group) were found to be near their normal values. Basic pancreatic trypsin inhibitor (BPTI, 58 residues) is also the protein of which pK values were all determined in the pH range 0–12 using 1D ^{1}H and ^{13}C NMR (Snyder et al., 1976; Brown et al., 1976, 1988; Richarz & Wüthrich, 1978). It is essential to obtain the experimental pK values of *all* the ionizable groups in a protein. From these values, the effective charge of each ionizable group is calculated as a function of pH. The resulting charge distribution is subsequently used to calculate the electrostatic potential on the surface of the protein to understand substrate–protein and protein–protein interactions (Matthew et al., 1985) or to analyze hydrogen exchange with solvent (Delepierre et al., 1987). There are various models for electrostatic effects in proteins which enable one to calculate theoretical pK values of the ionizable groups (States & Karplus, 1987). Thus, the experimental pK values can be used to check these models (Bashford & Karplus, 1990).

Detailed Characterization of pH-Induced Conformational Changes of Proteins. The pH-induced chemical shifts of protons reflect sensitively the local conformational change of proteins which is accompanied by the change of the electrostatic properties of the proteins (Wüthrich, 1986). Once all observable proton resonances are assigned to specific amino acid residues, these protons can be used as probes to localize the part of the proteins in atomic resolution where pH-induced conformational change occurs. In the present paper, we characterized the conformational change of mouse EGF in a slightly basic solution induced by deprotonation of the N-terminal α-amino group. Although the current interpretation of the results is rough, i.e., at a residue level, we expect that a detailed description of the conformational/electrostatic changes will be possible by analyzing the experimental chemical shift changes by reference to computed chemical shifts including anisotropic effects such as ring current shifts and carbonyl shifts (Dalgano et al., 1983).

Future Application of 2D NMR Titration. The activities of many enzymes vary with pH, reflecting the ionization of acid–base groups at the active site. By examining the pH dependence of k_{cat}, or k_{cat}/K_M, we can obtain the pKs of the enzyme–substrate complex, or those of the free enzyme and free substrate, respectively (Fersht, 1977). If the pK of each ionizing group is known, then we can identify the catalytic groups/residues, compared with the pKs of the enzyme re-

action. Thus, the p*K* determination of ionizable groups is quite important to understand the molecular mechanism of enzymes. There are several methods to determine the p*K*s of ionizable groups in proteins (Fersht, 1977). However, all of them have difficulty in assigning the experimental p*K*s to the specific ionizable groups. It is only the 2D NMR titration experiment that solves this problem. We are now studying the microbial ribonuclease family using 2D NMR titration to identify catalytic residues.

In conclusion, 2D NMR pH titration experiments need large amounts of machine time and analytical work but produce valuable information on the structure and dynamics of proteins.

Registry No. EGF, 39319-43-2.

REFERENCES

Bashford, D., & Karplus, M. (1990) *Biochemistry 29*, 10219–10225.

Bax, A., & Davies, D. G. (1985) *J. Magn. Reson. 65*, 393–402.

Brown, L. R., De Marco, A., Wagner, G., & Wüthrich, K. (1976) *Eur. J. Biochem. 62*, 103–107.

Brown, L. R., De Marco, A., Richarz, R., Wagner, G., & Wüthrich, K. (1978) *Eur. J. Biochem. 88*, 87–95.

Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 285–287.

Carpenter, G., & Cohen, S. (1979) *Annu. Rev. Biochem. 48*, 193–216.

Dalgano, D. C., Levine, B. A., & Williams, R. J. P. (1983) *Biosci. Rep. 3*, 443–452.

Delepierre, M., Dobson, C. M., Karplus, M., Poulsen, F. M., States, D. J., & Wedin, R. E. (1987) *J. Mol. Biol. 197*, 111–130.

Ebina, S., & Wüthrich, K. (1984) *J. Mol. Biol. 179*, 283–288.

Fersht, A. (1977) in *Enzyme Structure and Mechanism*, Freeman, Reading, U.K.

Folkers, P. J. M., Clore, G. M., Driscoll, P. C., Dodt, J., Köhler, S., & Gronenborn, A. M. (1989) *Biochemistry 28*, 2601–2617.

Honig, B., & Hubbell, W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5412–5416.

Jardetzky, O., & Roberts, G. C. K. (1981) in *NMR in Molecular Biology*, Academic, New York.

Klevit, R. E. (1985) *J. Magn. Reson. 62*, 551–555.

Kohda, D., & Inagaki, F. (1988) *J. Biochem. (Tokyo) 103*, 554–571.

Kohda, D., Go, N., Hayashi, K., & Inagaki, F. (1988a) *J. Biochem. (Tokyo) 103*, 741–743.

Kohda, D., Kodama, C., Kase, R., Nomoto, H., Hayashi, K., & Inagaki, F. (1988b) *Biochem. Int. 16*, 647–654.

Matrisian, L. M., Planck, S. R., & Magun, B. E. (1984) *J. Biol. Chem. 259*, 3047–3052.

Matthew, J. B., Gurd, F. R. N., Flanagan, M. A., March, K. L., & Shire, S. J. (1985) *CRC Crit. Rev. Biochem. 18*, 91–197.

Montelione, G. T., Wüthrich, K., Nice, E. C., Burgess, A. W., & Scheraga, H. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8594–8598.

Montelione, G. T., Wüthrich, K., Nice, E. C., Burgess, A. W., & Scheraga, H. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5226–5230.

Richarz, R., & Wüthrich, K. (1978) *Biochemistry 17*, 2263–2269.

Savage, C. R., Jr., & Cohen, S. (1972) *J. Biol. Chem. 247*, 7609–7611.

Savage, C. R., Jr., Inagami, T., & Cohen, S. (1972) *J. Biol. Chem. 247*, 7612–7621.

Shrager, R. I., Cohen, J. S., Heller, S. R., Sachs, D. H., & Schechter, A. N. (1972) *Biochemistry 11*, 541–547.

Snyder, G., Rowan, R., Karplus, S., & Sykes, B. D. (1975) *Biochemistry 14*, 3765–3777.

States, D. J., & Karplus, M. (1987) *J. Mol. Biol. 197*, 131–140.

States, D. J., Haberkorn, R. A., & Reuben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.

Tanford, C., & Kirkwood, J. G. (1957) *J. Am. Chem. Soc. 79*, 5333–5339.

Wüthrich, K. (1986) in *NMR of Proteins and Nucleic Acids*, pp 130–161, John Wiley and Sons, New York.