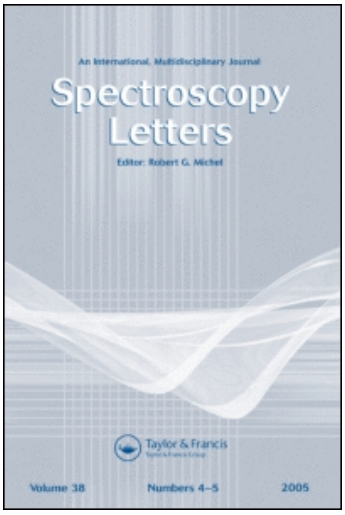

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pH-Induced Changes in the Secondary Structure of Cytochrome c: an Infrared Spectroscopic Study

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pH-Induced Changes in the Secondary Structure of Cytochrome c: an Infrared Spectroscopic Study

***Key words*: Fourier transformed infrared spectroscopy; cytochrome c; pH; secondary structure**

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Abstract

The infrared spectra of 3mM solutions of horse heart cytochrome c were recorded as a function of pD(corresponding to acid high spin form, acidic low spin form, native form, lysine form, the so-termed "strained lysine form" as well as the so-termed "A state" of cytochrome c). An analysis of the pH-induced changes in the secondary structure was performed based on changes in the

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conformation-sensitive amide I bands of this protein. In lysine and strained lysine forms, the contents of random structure increase at the expense of α-helix. In acidic low spin form(pH4-2.5), cytochrome c was almost unfolded. The proportion of α-helix had a substantial decrease while the contents of both random structure and 3_{10} -helices got a rise. In acidic high spin form(when pH is below 2.5), a further decrease of pD gave no rise to a continued unfolding in cytochrome c but made it refold to the "A state" with properties similar to those of a molten globular state. Adding enough KCl to the cytochrome c solution(pD2.2 with 0.5MKCl and pD3.0 with 1.5MKCl) in which cytochrome c was nearly fully unfolded also led to the formation of A state of cytochrome c. The compositions of all types of secondary structures in A state were evaluated. The behavior of 3_{10} -helices in acidic high/low spin form indicated they might be intermediates between α-helices and random structure in acidic solution as proposed by Miick et al.

Introduction

Cytochrome c is known to exist in several pH- and salt-dependent conformational states with altered heme ligation(1-5). It is reported that there exists at least six different forms of ferricytochrome c depending on the pH of the protein solution. They are acid high spin form(below pH2.5), acidic low spin form(between pH2.5 and 4), native form(between pH4 and 9), lysine form(between pH9 and 11), the so-termed "strained lysine form"(between pH11 and 12.5) and hydroxide form(above pH12.5)(3). In lysine form and strained lysine form, the sixth ligand, Met80 is replaced by a lysine epsilon amino group ligand. In hydroxide form, the sixth ligand is thought to be the external hydroxide anion. It is also suggested that in acidic form of cytochrome c, the Met80 is displaced by a ligand supplied by a solvent molecule. A number of spectroscopic

methods have been used for probing the structure of this protein in native form, including NMR, Raman, CD, FT-IR spectroscopy, etc.(1-5,16-18, and 24). However, the secondary structures of this protein in other forms have not been quantitatively determined and remain unclear.

It has been reported that acidification at high ionic strength or high concentration of HCl leads to a globular acidic form of cyt *c*(A state), which resembles the native protein in terms of intrinsic viscosity and the CD spectrum in the far UV region, but its stability toward thermal unfolding is drastically reduced(6, and 26-28). The A state of cyt *c* has been studied by means of absorption spectra, circular dichroism, and resonance Raman spectra(26-32). But all these methods were not able to supply quantitative information about the secondary structure composition of cyt c in A state. Kuroda et al studied the stability of α-helices of cytochrome c in a molten globule state (35°C, pH2.0 and 0.5MKCl) by means of hydrogen-deuterium exchange and two-dimensional NMR spectroscopy(29), but they did not identify other types of secondary structures of cyt *c* in A state. Hence no composition of each type of secondary structure in A state has been quantitatively determined.

In recent years, Fourier transformed infrared (FT-IR) spectroscopy has emerged as an increasingly valuable tool for examining protein conformation changes(7,18). Some groups have used this technique for studying the conformation changes of proteins in the effect of pH, temperature, pressure and ligand binding, etc(8-9,11-14). A few other groups have studied the secondary structure of native form cyt *c* by FT-IR(16, 17, and 18). Byler et al first reported research on the secondary structure of native form cytochrome c by FT-IR, but they did not point out the band assignment of 3_{10}-helix(18). Dong A. et al. studied the secondary structure of native form cyt *c* by secondary derivative amide I infrared spectroscopy(16-17) and developed a convenient method for the effective subtraction of the spectrum of water vapor(16). However, the secondary structures of cyt *c* in other forms have not been quantitatively studied.

In this work, we investigated the secondary structure changes of cytochrome in the effect of pH and for the first time reveal the compositions of each type of secondary structure of cyt *c* in acidic high spin form, acidic low spin form, lysine form, the so-termed "strained lysine form" and the A state. Through the comparisons between the secondary structures of these forms, the possible folding paths of cyt c in acidic and basic solution were discussed. And interestingly enough, in acidic solution, the behavior of 3_{10}-helices in native state to unfolded state transition indicated they might be intermediates between α-helices and random structures, and this was in conformity with Miick's suggestion(25).

Experimental

Sample Preparation cytochrome c from horse heart (type VI) was purchased from Sigma Chemical Co. and was purified prior to use(15). The protein was dissolved in D_2O , kept at 55°C for 1 day to exchange all the labile protons, then lyophilized. This procedure was repeated twice. Then the sample was dissolved in 0.25ml D_2O. Protein concentration was 3mM. To avoid the interference of buffer ions, such as PO_4^{3-} (which will present infrared peaks in amide I band), we used pure D_2O as the solvent and added small amounts of 6MDCl and NaOD to change pH. At the same time, we added the same quantity of DCl and NaOD to equal volume D_2O(2.5ml), which was used as the corresponding control. Twelve samples were prepared using Orion pH Meter to adjust pH(isotope effect was not corrected), one with pH7.0 was dissolved in H_2O, nine with pD1.5, 2.0, 2.2, 3.0, 3.5, 4.0, 7.0, 9.5 and 11.5 in pure D_2O, and two at pD3, 2.2 in 1.5M KCl and 0.5M KCl D_2O solutions respectively, which were used to study the molten globule state of cyt *c*. Each sample had its corresponding control provided with the same condition as the sample.

Infrared spectra. Infrared spectra were recorded using a Nicolet 170SX FTIR spectrophotometer at room temperature. Each sample was assembled between

CaF_2 windows separated with a 50μm Teflon spacer. The instrument was purged with dry air overnight prior to measurements. After the insertion of the sample into the chamber, the dry air purging was done for 30 min prior to the initiation of each spectral measurement. The spectra of controls for subtraction were obtained immediately followed by determining spectra of protein solutions. For each spectrum, a 1200-scan interferograms were collected at the single beam mode with a $2cm^{-1}$ resolution from 4000 to 1000 cm^{-1}. The experiments at pD1.5, 3.0, 7.0, 9.5, 11.5 were repeated to ensure the reliability of the experimental results.

Data Treatments. The presence of water vapor bands and a poor signal-to-noise ration can also lead to artifacts that are easily mistaken for protein bands in the resolution-enhanced spectra. The subtraction of absorption bands due to liquid water and vaporized water in the atmosphere was done using Dong's criteria(16). All the final sample spectra were obtained from the measured spectra of samples subtracted those of corresponding controls. The fourth-derivative spectra were obtained by taking the first derivative four times and were used to verify the peak assignment of the deconvoluted spectra. Fourier self-deconvolution of the amide I bands was performed using a Lorentzian of $13cm^{-1}$ half-bandwidth and a resolution enhancement factor(k value of 2.4), which generally give optimal resolution enhancement without producing evidence of excessive deconvolution, such as side lobes or other artifacts(18). Following the iterative fitting of Gaussian curves to the deconvoluted spectra, the relative amounts of the different secondary structures were assigned to the protein by integrating the areas under the curves assigned to a particular type of secondary structure. Both the deconvolved and original spectra are curve-fitted to verify the reliability of curve-fitting.

Results and Discussions

The spectra of cyt c in H_2O and D_2O are shown in FIG.1. In H_2O, two main bands are seen in the spectra region between 1850-1350cm^{-1}: the amide I band

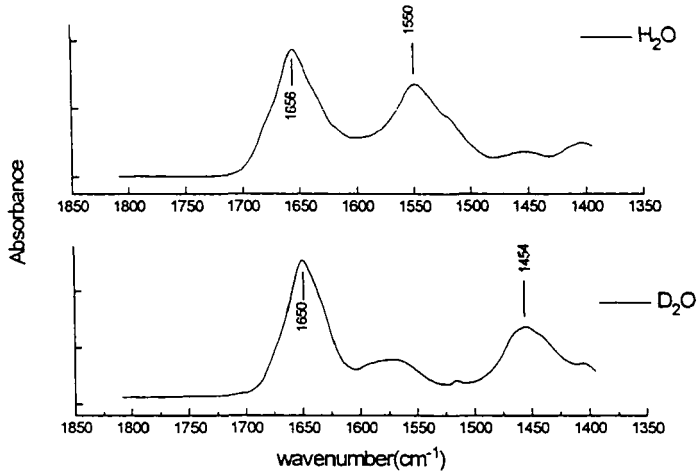

FIG.1. Infrared spectra of native form cyt c in H_2O(top) and D_2O(bottom).

around 1656cm^{-1} and amide II band around 1550cm^{-1}. Upon deuteration in D_2O, the accessible N-H groups will undergo H-D exchange. This can be seen in FIG.1(bottom spectrum), the amide I band shiftes to about 1650cm^{-1}, and the amide II band to 1454cm^{-1}. The rate and extent of hydrogen-deuterium exchange in proteins may be obtained by measuring the decrease in intensity of the amide II band near 1550cm^{-1}(10). The absence of the bands around 1550cm^{-1} in FIG.1(bottom spectrum) and 3300cm^{-1}(amide A band, essentially N-H stretching, not shown in the figure) indicates the complete H-D exchange has occurred. The bands still remaining between 1600-1500cm^{-1}(FIG.1, bottom spectrum) are due to amino acid side-chain absorptions.

The amide I band contours are the summation of several underlying component bands whose frequencies reflect different conformations of cyt c. These hidden bands can be visualized by resolution enhancement techniques. The deconvoluted and derivative spectra exhibit an essentially flat base line in the

1760-1850cm^{-1} region(not shown), suggesting that bands due to either excessive noise or water vapor do not contribute significant intensity to the resolution-enhanced spectra. FIG.2 shows the deconvolved and fourth-derivative spectra of native form cytochrome c together with its original spectrum. Ten bands are resolved in the fourth-derivative spectrum, their positions being consistent with the corresponding bands in the deconvolved spectrum. FIG.3 shows all the deconvolved spectra at different pD. It can be seen that for each spectrum, the position of each peak remains unshifted while its relative height varies with the pH. The quantitative contribution of each band to the total amide I contour may be obtained by the curve-fitting procedures. Eight bands between 1700 and 1620 cm^{-1} are considered to be due to the vibrations of the peptide bond, and the two bands shown below 1620cm^{-1}, although included in the curve-fitting procedure, are due to side-chain vibration(19). The results of curve fitting to both the deconvolved and the original experimental spectra agree well with each other. Some of them are shown in FIG.4 and FIG.5. The assignment of the individual bands to secondary structure elements is essentially based on FT-IR data published recently(16-17, 18). In D_2O, the amide I band frequency assignments for secondary structures available from previous studies are as follows: α-helix (1650±2cm^{-1}), β-extended chain (multiple bands between 1635 and 1620cm^{-1}), turns (multiple bands between 1685 and 1665 cm^{-1}), and random structure (1640±2cm^{-1}). The wavenumber of each of the above secondary structures is approximately 5 cm^{-1} lower than the corresponding value in H_2O. In our study, the band at 1650cm^{-1} was assigned to α-helix. The bands at 1630 and 1624cm^{-1} were assigned to β-extended chain(16, 17, and 20). Cyt *c* has little β-sheet, but has some extended chain around the heme. The component at 1640cm^{-1} was assigned to the random structure. The components found at 1666, 1673, and 1680cm^{-1} in D_2O were assigned to turn. The last band located at 1660 cm^{-1} offers a more difficult assignment. The high resolution crystallographic study and recent NMR study shows that there are about 8% 3_{10}-helices in horse heart cytochrome

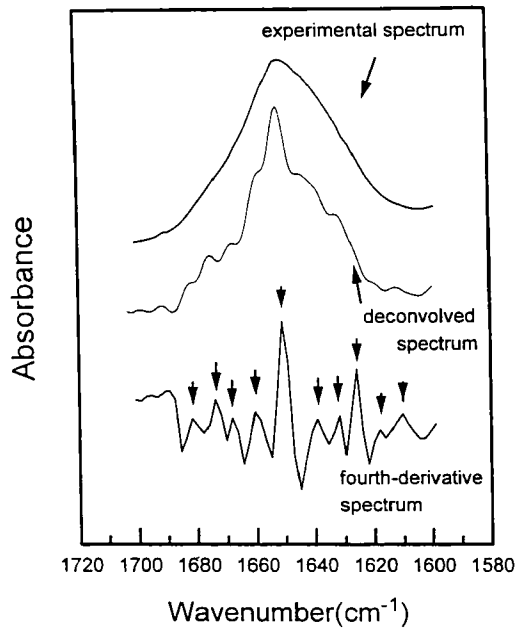

FIG.2. Experimental spectra and resolution enhancement spectra of native form cyt c in D_2O.

c(23, 33). As they are not very common, 3_{10}-helices have not been studied very well by using the infrared spectroscopy(7). Synthetic aminobutyric acid-containing peptides, known to form 3_{10}-helices, show strong amide I bands at 1662-1663 cm^{-1}(21). Other experimental and theoretical studies also indicate that distorted helical structures such as 3_{10} or αII helices exhibit amide I frequencies which are higher than those observed for α-helices due to weaker hydrogen bonding(21, 22). Therefore, we can reasonably assign the 1660cm^{-1} band to 3_{10}-helices.

TABLE 1 lists the contents of each secondary structure element of cyt c in different pD. The changes of the proportions of each secondary structure versus pD are clearly visualized in FIG.6. In native form, the contents of the various

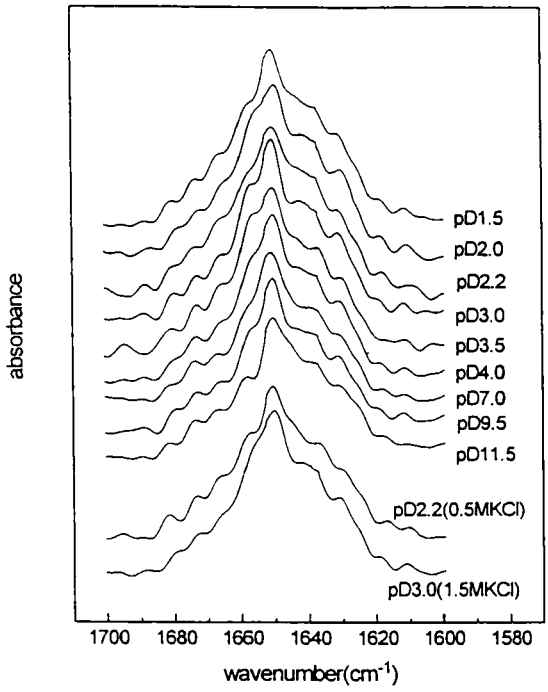

FIG.3. Deconvolved spectra of each form of cyt c at pD 11.5, 9.5, 7.0, 4.0, 3.5, 3.0, 2.2, 2.0, 1.5 and pD2.2, 0.5MKCl; pD3.0, 1.5MKCl. (Deconvolution parameters: half-bandwidth of 13cm^{-1}, resolution enhancement factor k of 2.4).

secondary structures estimated by FT-IR agree well with those determined by X-ray crystallography (23). In lysine form, the secondary structure is very similar to that of native form, only about 7% of α-helix turning into the random structure. This indicates that during the transition of cyt c from native form to lysine form, only local structure is unfolded, while in strained lysine form, the content of random structure has such a substantial increase that it overweighs that of α-helices. This indicates the strained lysine form is a quite unfolding state of cyt c. From FIG.6, we can find that during the process in which cyt c changes from

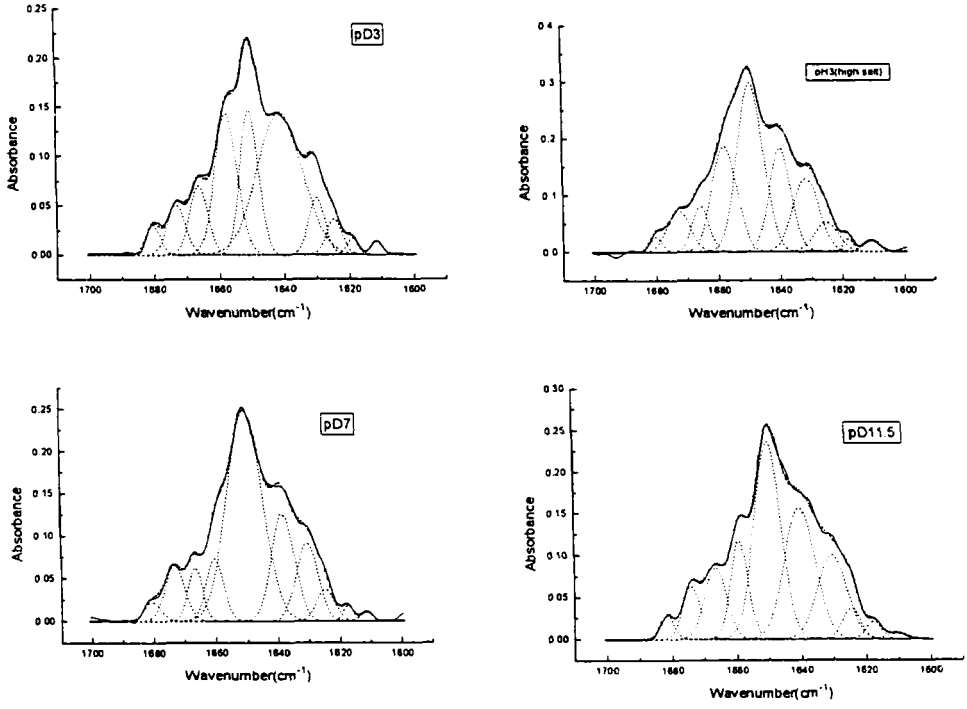

FIG.4. The curve-fitted deconvolved amide I spectra of cyt c at pD3.0, pD3.0(1.5MKCl), pD7.0, pD11.5. Curve-fitting was carried out as described under Experimental.

native form to lysine form and strained lysine form, the proportion of random structure increases at the expense of α-helices while the fractions of other types of secondary structures(turn, extended chain and 3_{10}-helix) remain nearly the same. Therefore we could suggest that in neutral and basic solution(pD4-11.5), the possible folding/unfolding path of cytochrome c on the effect of pH is: α-helix ⇔ random structure.

In acidic low spin form, things are more complicated. During pD4-3, a great change takes place in the secondary structure of cytochrome c: from almost native form-like to nearly fully unfolded. In pD3.0, only 17% α-helices are retained, the

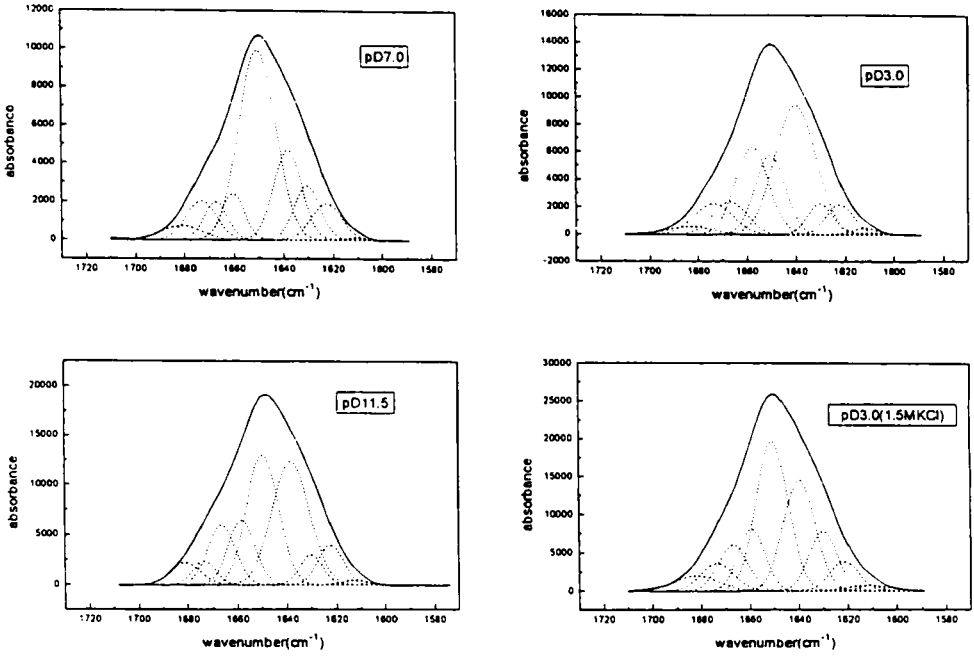

FIG.5. The curve-fitted experimental amide I spectra of cyt c at pD3.0, pD3.0(1.5MKCl), pD7.0, pD11.5. Curve-fitting was carried out as described under Experimental.

content of random structure rising to 39%, and the proportion of 3_{10}-helices also increasing to 19%. When pD is further lowered to be in acidic high spin form, cyt *c* does not continue unfolding but refold. In pD2.2, the contents of α-helices rises back to 27% while the proportions of both random structure and 3_{10}-helices decrease. A further addition of DCl leads cytochrome c to the "A state" which resembles a molten globular state(26-27). In pD1.5, the contents of α-helix increase to about 33% while the fractions of random structure and 3_{10}-helices decrease to 25% and 12% respectively. In Goto et al's study on acid-induced folding of proteins(27), the midpoint concentration of HCl induced native form

TABLE 1. The fractions of each type of secondary structure versus pD

		secondary structure (%)[a]				
pD		α-helix	3_{10}-helix	extended structure	turn	random
acidic high spin form	1.5	33	12	14	16	25
	2.0	28	16	16	14	26
	2.2	27	16	14	16	27
acidic low spin form	3.0	17	19	10	15	39
	3.5	35	14	15	16	20
native form	4.0	43	9	15	14	19
	7.0	48	8	14	14	16
lysine form	9.5	39	9	14	16	22
strained lysine form	11.5	29	9	14	17	31
A state	2.2(0.5 M KCl)	36	12	14	17	21
	3.0(1.5 M KCl)	32	13	16	17	22

[a]Percent areas obtained by curve-fitting: estimated error ±5%

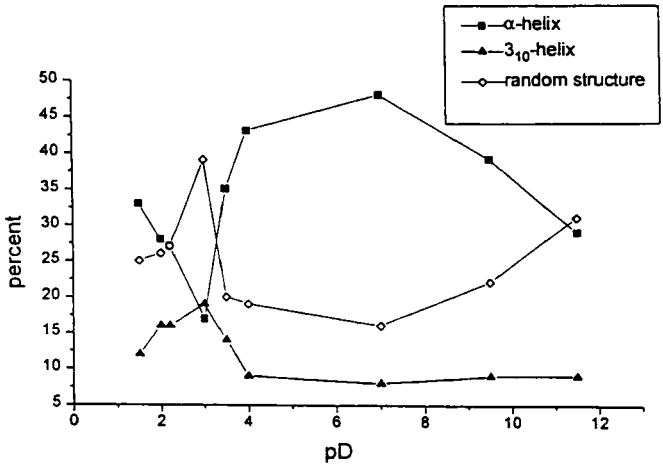

FIG.6. The fraction of each type of secondary structure changes versus pD.

and A state transition is 85mM. And 100mM HCl(about pH1.0) will lead most proteins to A state. This is in consistency with our results.

Not only the high concentration of HCl but also acidification at high ionic strength will lead proteins to A state. At pD3.0 , cyt *c* is nearly fully unfolded. When adding 1.5M KCl, the amount of α-helices rises from 16% to 32%. This indicates A state has been formed. At pD2.2, things are almost the same except for the fact that the contents of α-helices have risen from 27% to 36%. Our study of A state of cyt c is in agreement with Goto et al's results(26-27). But they focused on the condition of the transition from native state to A state whereas we are interested in the compositions of each type of secondary structure in A state and their changes during the transition. A structural model for the A-state has been proposed by Jordan T etc.(30) using the resonance Raman spectroscopy in which there are about 35% α-helices. In our study, the proportion of α-helices in A state is near 35%, which supports Jordan's proposal.

From TABLE 1 and FIG.6, it is obvious that in acidic high or low spin form, the content of 3_{10}-helices does not remain constant; it changes in the same way as random structure. When the content of α-helices decreases, the content of 3_{10}-helices rises together with that of random structure; on the other hand, when the content of α-helices increases, the content of 3_{10} helices decreases as does the content of random structure. So in the acidic solution, the possible folding/unfolding path of cyt *c* on the effect of pH is different from that in the neutral and basic solution. Miick (25) suggested that 3_{10}-helix may be a long-lived intermediate between the two terminal states of α-helix and random coil. We thus formulate the possible folding path of cyt c on the effect of pH in acidic solution is: α-helix $\Leftrightarrow$ 3_{10}-helix $\Leftrightarrow$ random structure.

Copeland et al(24) reported ultraviolet resonance raman spectra of cyt c at pH 1.5, 7, 10, and 13. They found the amide I peak (whose frequency was consistent with a dominant contribution from α-helical regions) broadened when native protein was converted to the low- or high-pH forms(pH1.5 and 10),

reflecting some disordering of the polypeptide chain, but the peak frequency did not shift, thereby establishing that the α-helical regions were not completely unfolded. Raising the pH to 13 did produce a frequency upshift, reflecting helix refolding. Their qualitative results are consistent with ours. However, they did not give as detailed information about the secondary structure in these forms as we did.

To our knowledge, this paper presents the first systematic FT-IR spectroscopic study of the several forms of cytochrome c depending on pH. We quantitatively determined the contents of each type of secondary structure in each form and in A state of cyt *c*, and suggest different folding paths in acidic and basic solutions. Our results also support Miick's suggestion that 3_{10}-helix is mainly a long-lived intermediate between the two terminal states of α-helix and random coil.

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