Conformational Study of Glutathione by NMR

Shizuo FUJIWARA, Grazyna FORMICKA-KOZLOWSKA,* and Henryk KOZLOWSKI*

Department of Chemistry, Faculty of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113

**Institute of Chemistry, University of Wrocław, Wrocław, Poland*

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Proton NMR spectra of glutathione have been analyzed over the whole pH region. The spectra of the glutamic acid residue are of the ABCDX type at pHs below 2.5, of the AA'BB'X types in the pH region 2.5—9, and of the ABCC'X type at pHs higher than 9. For the cysteine part, spectra of the ABC type have been observed at pHs higher than 8, which change to spectra of the A_2B type at pHs lower than 7. On the basis of these results the conformation of the glutathione molecule in solution has been discussed in detail.

The NMR technique has been successfully used in the conformational study of various amino acids.[1-9] A similar study of more complex molecules like polypeptides is usually complicated by poorly resolved spectra and a large number of coupled protons.

A fundamental and manifold interest in the recognition of the chemical, physical, and biological properties of peptides has occasioned our attempts to establish the structure of glutathione in solution by means of NMR.[10-14] Knowledge of the conformation of glutathione should help us in better understanding some metal-glutathione interaction, which is the further aim of our study.

Experimental

Materials. The glutathione was purchased from Wako Pure Chemical Industries, and the D_2O, from E. Merck. The pHs of the solutions were controlled by adding a concentrated DCl or NaOH solution in D_2O, and measured with a Toa Dempa pH-meter, model HM-5A. The concentration of glutathione in the solutions was 0.1 M. The chemical shifts are reported in Hz relative to internal tetramethylammonium (TMA). The NMR spectra were recorded with a JNM PS-100 spectrometer operating at 100 MHz.

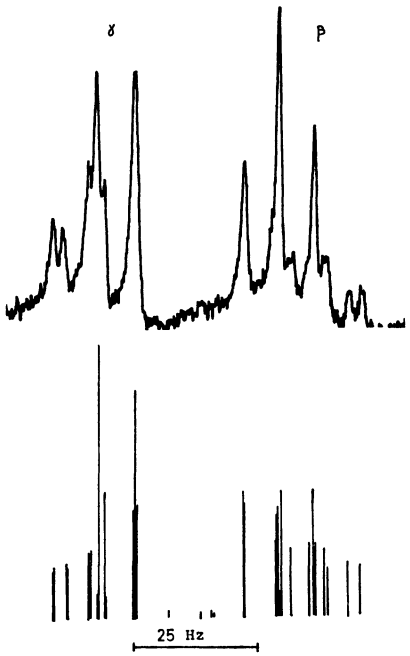

Fig. 1. Experimental and calculated spectra of γ and β protons of the glutamic acid residue at pH 0.7.

Analysis of Spectra. *Glutamic Acid Residue Spectra*: Five protons of the glutamic-acid part give spectra of the ABCDX type at pH lower than 2 (Fig. 1), of the AA'BB'X type in the pH range of 2.51—8.05 (Fig. 2), and of the ABCC'X type at pH higher than 9 (Fig. 3). Here ABCDX, AA'BB'X, or ABCC'X refers to the β, β', γ, γ', α protons (Fig. 4). In the pH region of 2.5—8.05 the AA'BB' pattern for the β and γ protons was confirmed by the decoupling of the α proton. An analysis of the spectra observed at a certain pH was carefully carried out with a set of input data which had been obtained in the analysis of spectra observed at a slightly different pH. It has been found that, in order to reproduce a calculated spectrum which is in good agreement with the

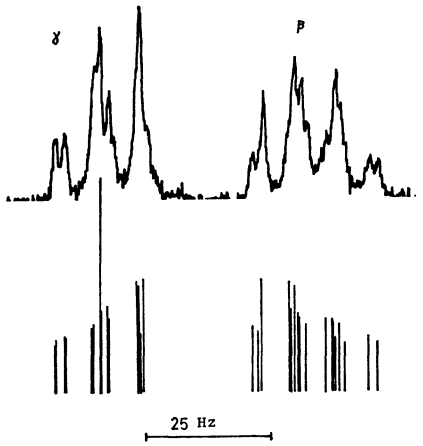

Fig. 2. Experimental and calculated spectra of γ and β protons of the glutamic acid residue at pH 4.5.

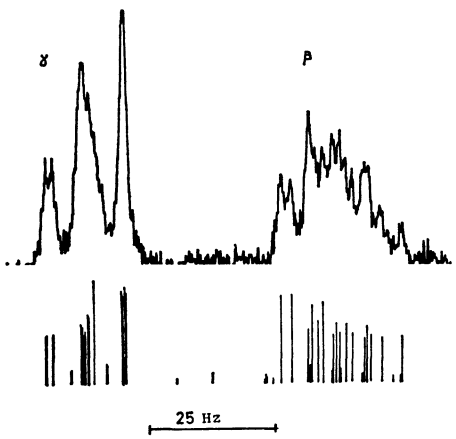

Fig. 3. Experimental and calculated spectra of γ and β protons of the glutamic acid residue in 2 M NaOH.

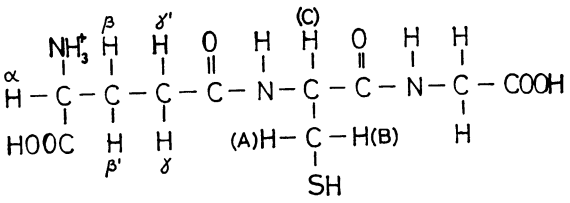

Fig. 4. Glutathione molecule and notation of the protons.

TABLE 1. NMR PARAMETERS OF THE GLUTAMIC ACID RESIDUE

pH	$\Delta\nu_{\beta\beta'}$	$\Delta\nu_{\gamma\gamma'}$	$J'=J_{\beta\gamma}=J_{\beta'\gamma'}$	$J=J_{\beta\gamma'}=J_{\beta'\gamma}$	$J_{\beta\alpha}$
2 M NaOH	9.75	0	10.5	5.55	6.56
12.8	9.8	0	10.5	5.6	6.6
9.58	3.8	0	9.2	6.45	6.4
9.1	1.6	0	8.8	6.9	6.4
8.05	0	0	8.5	7.15	6.34
7.75	0	0	8.5	7.05	6.30
4.5	0	0	8.4	7.1	6.34
2.95	0	0	8.5	7.2	6.3
2.51	0	0	8.3	7.15	6.39
2.02	2.7	2.55	7.95	7.3	6.55
1.2	5.2	4.65	7.55	7.35	6.52
0.7	5.4	4.9	7.45	7.35	6.55
6 M DCl	6.2	5.1	7.6	7.4	6.54

All the NMR parameters are given in Hz.

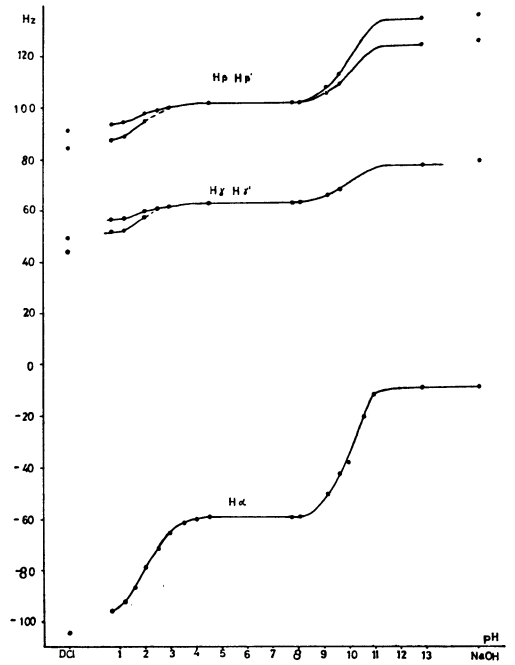

Fig. 5. pH dependence of the protons of glutamic acid residue relative to TMA.

observed one, the chemical shifts and spin-coupling constants have to be correct within ± 0.2 Hz. J_{gem} was assumed to be -15.0 Hz, and $J_{\alpha\gamma}=J_{\alpha\gamma'}=0$, which was confirmed by the decoupling of the β protons. All the NMR parameters for the glutamic acid residue over the whole pH region are given in Table 1 and Fig. 5.

Cysteine and Glycine Residues. The NMR spectra of the cysteine residue are of the ABC type. Below pH=8, however,

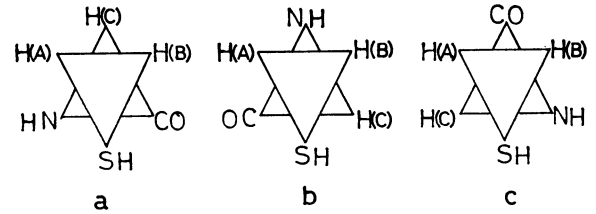

Fig. 6. Three rotational isomers of cysteine.

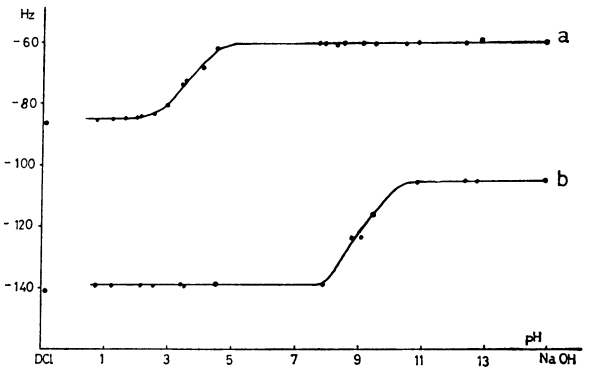

Fig. 7. pH dependence of the glycine protons (a) and C proton of cysteine (b) relative to TMA.

TABLE 2. NMR PARAMETERS OF THE CYSTEINE RESIDUE

pH	$\Delta\nu_{C-TMA}$	J_{AB}	J_{AC}	J_{BC}	$\Delta\nu_{AB}$
2 M NaOH	105.4	−13.2	7.88	5.12	11.1
12.95	103.3	−13.0	8.1	4.85	11.4
12.8	105.0	−13.1	7.9	5.00	11.0
12.4	105.1	−13.0	8.1	4.9	11.5
10.9	105.9	−13.1	8.05	4.85	11.2
9.5	116.6	−13.5	7.96	4.9	10.7
9.1	123.7	−13.8	7.8	4.9	8.2
8.8	123.9	−14.1	7.6	5.0	9.0

All the NMR parameters are given in Hz.

the spectra are very poorly resolved and have to be analysed as the A_2B type. In this pH region, $J_{AC}=J_{BC}=6.3$ Hz. Using the notation "ABC" given in Fig. 6, the NMR parameters are given in Table 2, where the chemical shift of the A proton is taken to be in a higher field than that of the B proton. The pH dependence of the chemical shifts of the C proton of the cysteine and CH_2 protons of the glycine are shown in Fig. 7. The CH_2 protons of the glycine give a singlet line due to the exchange by deuterium of the NH proton of the neighboring peptide nitrogen,** and there is a small difference in the chemical shifts of the two protons. To confirm the pH dependence of the chemical shifts, the microscopic ionization constant (Fig. 8) were calculated using a method described by Rabenstein[10] (Table 3).

Rotational Isomerism. In order to discuss the conformation of the entire glutathione molecule, the populations of the rotamers for cysteine and glutamic acid residues were calculated separately.***

** In the case of the H_2O solution, above pH 7.5 the CH_2 protons of glycine also give a singlet as a result of the averaging of conformations due to the fast exchange of peptide protons.

*** The population of rotational isomers was obtained by following the procedure originally used by Pachler.[1] The scope and limitations of this method, especially in the case of simple amino acids, have been discussed previously.[2]

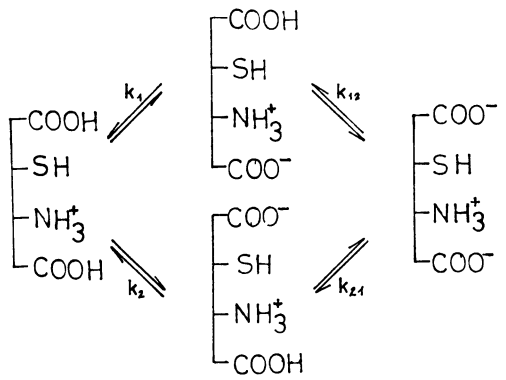

Fig. 8. Microscopic ionization scheme for carboxyl groups of the glutathione.

Table 3. Microscopic ionization constants for the dissociation of the carbonyl group

	This work	Ref. 10
pk_1	2.05±0.1	2.09±0.05
pk_2	3.14±0.05	3.12±0.05
pk_{21}	2.33±0.05	2.33±0.01
pk_{12}	3.42±0.1	3.36±0.1

Rotamers for Glutamic Acid Residue. In this part of glutathione, nine rotamers have to be considered (Fig. 9). From the experimental data, $J_{\alpha\gamma}\cong 0$. Thus, it is not possible to find the relative conformations of the α and γ protons from the J value. We will calculate the populations of the I, II, and III rotamers (Fig. 10a) and, independently, the populations of 1, 2, and 3 rotamers (Fig. 10b).

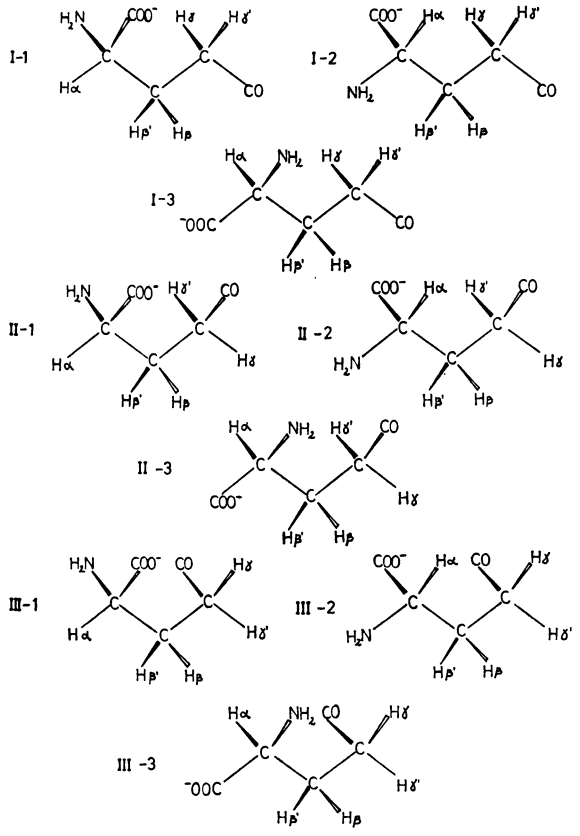

Fig. 9. Nine rotational isomers of the glutamic acid residue.

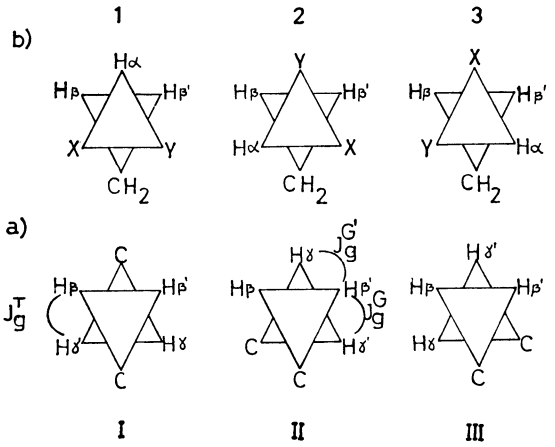

Fig. 10. The partial rotamers of glutamic acid:
a) for β and γ carbons
b) for α and β carbons.
X and Y are the carboxyl and amino groups, respectively.

Rotamers I, II, and III. We will approximate this unit in the glutamic residue as a substituted ethane. For the estimation of an average vicinal coupling constant, it may be possible to use the Abraham and Pachler empirical equation;[16]

$$J_{av} = 17.96 - 0.796 \sum_{i=1}^{6} E_i \qquad (1)$$

where E_i is the Huggins electronegativity of atoms attached to the ethyl carbons. From this equation, $J_{av}\cong 7.1$. Equation 1, however, is only a general expression for the relation between the electronegativity and J_{vic}, and it seems more reasonable to use the experimental data for the estimation of J_{av}. At pH 0.7, all four J values are almost equal to each other within the limits of experimental error, and $J_{av}=7.41$. Because there are two different sets of J values, we rewrite: $J_{\beta\gamma}=J_{\beta'\gamma'}=J'$ and $J_{\beta\gamma'}=J_{\beta'\gamma}=J$, and, if $N=J+J'$ and $L=J-J'$, we obtain another expression for the J_{av};

$$\begin{aligned} J_{av} &= 1/3(3/2N+1/2L) \\ &= 1/3(2J+J') \\ &= 1/3(J_t+2J_g) \end{aligned} \qquad (1')$$

and $J_{av}=7.43$ Hz if $(3/2N+1/2L)=22.3$. For further discussion, $J_{av}=7.4$ Hz will be used. The vicinal coupling constants, $J_{\beta\gamma}$, $J_{\beta\gamma'}$, $J_{\beta'\gamma}$, and $J_{\beta'\gamma'}$ for this system (Fig. 10a) are given by the following equations:

$$\begin{aligned} J_{\beta\gamma} &= p_I J_t + p_{II} J_g^G + p_{III} J_g^G \\ J_{\beta'\gamma'} &= p_I J_t + p_{II} J_g^G + p_{III} J_g^G \\ J_{\beta\gamma'} &= p_I J_g^T + p_{II} J_t + p_{III} J_g^{G'} \\ J_{\beta'\gamma} &= p_I J_g^T + p_{II} J_g^{G'} + p_{III} J_t, \end{aligned} \qquad (2)$$

where p_I, p_{II}, and p_{III} are the populations of the I, II, and III rotamers and J_g^T, J_g^G, and $J_g^{G'}$ are as defined in Fig. 10a. From the Eq. 2 one can see directly that, for any values of p_I, p_{II}, and p_{III}, $J_{\beta\gamma}=J_{\beta'\gamma'}$. This results is in agreement with the results of the analysis. Also from these results, $J_{\beta\gamma'}=J_{\beta'\gamma}$; hence, $p_{II}=p_{III}$. The fact that the populations of both rotamers of the gauche form are equal to each other agrees with the results obtained for simple 1,2-disubstituted ethanes.[17,18] This appears to justify our assumption that the $-CH_2-CH_2-$ unit in glutamic acid can be considered as a substituted ethane.

Equation 2 may be simplified to this form:

$$J' = J_{\beta\gamma} = J_{\beta'\gamma'} = p_I J_t + 2p_{II} J_g^G$$
$$J = J_{\beta\gamma'} = J_{\beta'\gamma} = p_I J_g^T + p_{II}(J_g^{G'} + J_t) \quad (2')$$

To estimate populations, it is necessary to have the values of J_g^T, $J_{g'}^G$ and $J_g^{G'}$ ($J_g^T > J_g^G > J_g^{G'}$ [17]).

Table 4. Populations of the I, II, and III rotamers of the glutamic acid residue

pH	$\bar{p}_I$	$\bar{p}_{II}$	Δp_{II}	$3/2N+1/2L$
2 M NaOH	0.60	0.20	0.01	21.60
12.8	0.59	0.20	0.01	21.77
9.58	0.48	0.26	0	22.11
9.1	0.42	0.29	0	22.56
8.05	0.40	0.30	0.01	22.78
7.75	0.40	0.30	0	22.53
4.5	0.40	0.30	0.01	22.58
2.95	0.39	0.30	0.01	22.86
2.51	0.39	0.30	0.01	22.58
2.02	0.36	0.32	0.01	22.64
1.2	0.34	0.33	0	22.25
0.7	0.34	0.33	0	22.20
6 M DCl	0.34	0.33	0	22.32

From Table 4 it can be seen that, with an increase in pH, the J' value also increases. According to Equations 2', this result implies that there is an increase in the population of a trans isomers (p_I). Thus, if the J_g^T values is sufficiently higher than that of J_g^G and $J_{g'}^{G'}$ the values $(3/2N+1/2L) = (J'+2J)$ should steadily increase with an increase in p_I. In the present case, these values are almost constant at all pH values, equal to 22.2 ± 0.6 Hz. The deviation may be attributed to the experimental error. However, the deviation of this range may be caused by a small cahnge in the nature of the medium, such as the dielectric field in the solution, or the dissociation of functional groups of the glutathione. $J_{g'}^T$, $J_{g'}^G$, or J_g^G should be constant throughout the pH rang examined. Therefore, it seems reasonable to put one J_g value into Eq. 2'. By combining Eqs. 2' and 3:

$$p_I + 2p_{II} = 1 \quad (3)$$

the following expressions can be obtained:

$$\text{from } J': \quad p_I = (J'-J_g)/(J_t-J_g), \quad p_{II} = 1/2(J_t-J')/(J_t-J_g), \quad (4)$$

$$\text{from } J': \quad p_I = (J_t+J_g-2J)/(J_t-J_g), \quad p_{II} = (J-J_g)/(J_t-J_g). \quad (4')$$

In order to determine J_t and J_g in addition to J_{av}, it is necessary to assume the J_t/J_g ratio, for which we will take a common value of 5.2[1,2,9,20] ($J_t/J_g = 5.6$ for pure ethane[19]) for all α-amino acids. The result is that $J_t = 16.03$ Hz and $J_g = 3.08$ Hz. From Eqs. 4 and 4' we can determine two sets of p_I and p_{II} values, using the J' or J spin-coupling constant. The very good agreement between the two sets of p_I and p_{II} populations confirms that, at least for a qualitative discussion, the present assumptions are sufficiently correct. The average values, $\bar{p}_I$ and $\bar{p}_{II}$, and the deviation of $\bar{p}_{II}$ from the values obtained using Figs. 4 and 4' are given in Table 4.† $J_{\alpha\beta}$ changes only slightly from about 6.3 Hz in the pH range 2.9—8.05 to about 6.6 Hz for pH values lower than 0.7 and higher than 11. Using the J_t and J_g values estimated for simple amino acids,[1,2,9] it is easy to find that, in the region of pH 2.9—8.05, all three rotamers, 1, 2, and 3 (Fig. 10b), have the same population. In other pH regions there is some excess of the 2 and 3 rotamers over the 1 rotamer, the populations being 0.36, 0.36, and 0.28. The 2 and 3 rotamers cannot be distinguished from one another because the observed spectrum is of the A_2X type. (We can determine only the average value of $J_{\alpha\beta}$).

Cysteine Residue. Using $J_g = 2.6$ Hz, $J_t = 13.6$ Hz, and $J_{av} = 6.3$ Hz, the populations of the three *a*, *b*, and *c* rotamers (Fig. 6) can be obtained (Table 5). However, it is not possible to distinguish the A and B protons in this experiment. Furthermore it is not certain which rotamer, *b* or *c*, is more stable. In the pH region where poorly resolved spectra are observed, $J_{AC} = J_{BC} = 6.3$ Hz and $a = b = c = 1/3$.

Discussion

Glutamic Acid Residue. The I-1, 2, and 3 rotamers appear to be stable with respect to the interaction of the two terminal functional groups of this residue with the peptide linkage between glutamic and cysteine parts. In gauche isomers (II, III), the II-1 rotamer appears to be the least stable because of the electrostatic interaction of the carboxyl and ammonium group with the peptide backbone, especially with the carbonyl group. For a similar reason, the III-2 isomer seems to be less stable. At pH<2, the total population of the gauche form is large in comparison to the trans one (I), and this interaction with the peptide-bond group induces an observable difference in the populations of the 1, and 2, and 3 rotamers. It is also likely that there exists some difference in population of the II-2 and II-3 rotamers, and so on, but the fast interconversion between the two isomers of the gauche form (II and III) (*e.g.*, II-2—III-2) makes it impossible to distinguish them.††

The dissociation of the COOH group to COO^- which occurs between pH 0.7—2.95 makes this interaction stronger because of the excessive negative charge on the carboxyl group, and some increase in the population of the trans (I) form has been observed. In the pH range of 8—11, where the dissociation of the NH_3^+ group occurs, an additional negative charge on the glutamic terminal causes an other increase in the population of the trans (I) isomer, for the same reason. The rotamers stable at pH values higher than 11 are I-1, 2, and 3, and the gauche forms, II-2 and III-3. The least stable should be II-1 and III-1.

At pHs below 2.5 and pHs higher than 9, the unequal populations of the 1, 2, and 3 rotamers distinguish the chemical shifts of β and β', while at pHs below 2.5 the chemical shifts for γ and γ' protons can be differentiated. The interaction of the carboxyl and the ammonium groups with the γ and γ' protons is more effective in the gauche form of the unit considered, and at high pHs probably a smaller population of this form causes $\Delta_{\gamma\gamma'}$, to equal 0. To explain this, more accurate populations of the particular rotamers, II-1—III-3, have to be known.

Cysteine. Below pH 8, $a = b = c = 1/3$. When the

† The $\bar{p}$ values are the average values of the populations obtained from Eqs. 4 and 4'.

†† In this case, a more complex intercoversion between the 1, 2, 3 and I, II, III rotamers should be considered.

Table 5. Populations of the cysteine residue rotamers

pH	*a*	*b*(or *c*)	*c* (or *b*)
2 M NaOH	0.29	0.48	0.23
12.95	0.29	0.50	0.21
12.8	0.30	0.48	0.22
12.4	0.29	0.50	0.21
10.9	0.30	0.49	0.21
9.5	0.30	0.49	0.21
9.1	0.31	0.48	0.21
8.8	0.32	0.46	0.22
<8	$a \cong b \cong c \cong 0.33$		

SH group dissociates to S^-, the spectra are better resolved (ABC type) and a more detailed population of the three rotamers (*a*, *b*, and *c*) can be found (Table 5, Fig. 6). The smallest change of the population is observed for the a rotamer, where the population is decreased from 0.33 to about 0.29. This result means that the most stable rotamer after the dissociation of the SH group is the one in which the position of S is trans to the cysteine peptide nitrogen or the carbonyl peptide group.

From all these considerations it seems that a peptide linkage between glutamic acid and cysteine, and the interaction of the carbonyl group in the peptide linkage with the changes in the functional groups of the glutamic residue are important for the conformational equilibrium in solution. Thus, the most stable conformer after the complete dissociation of the peptide molecule is the one in which the COO^- and NH_2 groups of the glutamic acid residue are far from the peptide backbone of the glutathione.

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