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Side-Chain Motions in Peptides Bound to Elastase. NMR Relaxation Investigation on Carbon-13 Specifically Enriched Trifluoroacetyl-Tripeptide Inhibitors†

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ABSTRACT: Kinetic, ^{19}F NMR, and recently X-ray data have shown that CF_3CO-tripeptides and dipeptide anilides bind in a unique mode to elastase, the CF_3CO group interacting with a specific site on the enzyme. We report the results of an NMR study of the interaction with elastase of the two CF_3CO-tripeptides, CF_3CO-Ala_3 and CF_3CO-Lys-Ala_2, in which the N-terminal residue has been uniformly enriched with ^{13}C. We first show that neglecting cross-relaxation and cross-correlation effects should lead at 25 and 50 MHz to small errors in ^{13}C relaxation times T_1 of uniformly enriched molecules bound to elastase. Under these conditions, the model-free analysis [Lipari, G., & Szabo, A. (1982) *J. Am. Chem. Soc. 104*, 4546–4559] of the ^{13}C relaxation times of the two enzyme–inhibitor complexes obtained at two frequencies of observation by using the C_α carbon as a probe of the protein motion provides estimations of the generalized order parameters S^2 of each ^{13}C atom. These estimations allow calculation with a good precision of the rotation angles γ_{0i} about each $C_{i-1}C_i$ bond, selecting restricted multiple rotation as a model for the side-chain motion and assuming that the lowest energy conformation of the lysine side chain in solution is trans as observed in the crystal. Angles γ_{0i} once obtained have then been used to estimate the correlation times τ_i of rotation about each $C_{i-1}C_i$ bond, from a complete analysis of the experimental T_1. The results show that the $C_\beta H_3$ of [^{13}C]Ala is still freely rotating in the complex. In contrast the side chain of [^{13}C]Lys is found to be highly immobilized. Furthermore, the difference of orientation of the Ala and Lys side chains is suggested by the different chemical shift behaviors of the C_β of these amino acids during complexation. The similar affinity of both peptides is interpreted in light of these NMR data.

The peptide CF_3CO-Lys-Ala-NH-C_6H_4-*p*-CF_3 is a strong inhibitor of elastase. The structure of its complex with porcine pancreatic elastase has been recently determined at 2.5-Å resolution by X-ray crystallography (Hughes et al., 1982). This resolution is sufficient to observe close contacts between the peptide and the protein and to estimate the local mobilities through isotropic atomic temperature coefficients. On the other hand, enzyme kinetics and ^{19}F NMR have shown that CF_3CO-tripeptides and dipeptide anilides bind to the enzyme in a single mode involving the interaction of the trifluoroacetyl group at a specific site of the protein (Dimicoli et al., 1979, 1980). Their affinity for elastase critically depends, however, on the nature of each amino acid and also on the substituents

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on the anilide ring. The specific interactions of the enzyme with the various chemical groups of the inhibitor are thus important for the overall affinity.

^{13}C NMR is a powerful method for the study of such interactions in terms of local immobilization of the peptide inhibitors within the complexes as demonstrated theoretically (Lipari & Szabo, 1982; Levy & Sheridan, 1983) and experimentally (London & Avitabile, 1978; London et al., 1982). The large affinity of the CF_3CO-peptides for elastase is favorable for such investigations in solution. Two specifically ^{13}C-enriched inhibitors have been synthesized, CF_3CO-[^{13}C]Ala-Ala_2 and CF_3CO-[^{13}C]Lys-Ala_2, in which all carbons of the labeled amino acid are enriched in ^{13}C at 85%. The labeled inhibitors should thus permit the investigation of the effect of the nature and length of the side chain of the N-terminal amino acid on the internal mobility and indirectly on the affinity of the peptide for the enzyme.

The data have been analyzed in terms of ^{13}C dipolar relaxation using the "model-free" approach of Lipari & Szabo (1982). This theory is rigorous in the case where the ^{13}C relaxation depends only on a single directly bound proton. It was thus necessary to check its applicability to ^{13}C-enriched compounds in which cross-relaxation and cross-correlation effects on the ^{13}C relaxation may exist.

Theory

Analysis of the Dipolar Relaxation Times. The analysis of the ^{13}C relaxation times assuming a priori a microscopic model of the motion of the ^{13}C–H bond involves the simultaneous fitting to the data of the corresponding amplitude and frequency parameters. The model-free analysis of Lipari & Szabo (1982) is considerably simpler since in conditions of extreme narrowing for the internal motions, the relaxation times T_1 may be described by the following simple relation:

$$\frac{1}{T_1} = \frac{\mathcal{S}^2}{T_1^R} + C(1 - \mathcal{S}^2)\tau_e \qquad (1)$$

where C is a constant if the length of the ^{13}C–H bond is constant, T_1^R is the relaxation time of a ^{13}C rigidly attached to the macromolecule, and $\mathcal{S}^2$ and τ_e are the only amplitude and frequency effective parameters describing the motion which may be unambiguously obtained from NMR measurements. These parameters may thus be estimated explicitly through the values T_1 and $\tilde{T}_1$ of the relaxation time obtained at two frequencies according to the relations

$$\mathcal{S}^2 = \frac{1/T_1 - 1/\tilde{T}_1}{1/T_1^R - 1/\tilde{T}_1^R} \qquad (2)$$

$$\tau_e \frac{\hbar^2\gamma_C^2\gamma_H^2}{r_{CH}^6} = \frac{T_1^{-1}(\tilde{T}_1^R)^{-1} - \tilde{T}_1^{-1}(T_1^R)^{-1}}{T_1^{-1} - \tilde{T}_1^{-1} - (T_1^R)^{-1} + (\tilde{T}_1^R)^{-1}} \qquad (3)$$

$\mathcal{S}^2$ is in fact the long time limit of the autocorrelation function of the ^{13}C–H dipolar interaction in a frame bound to the enzyme and may be considered as a generalized order parameter since it satisfies $0 < \mathcal{S} < 1$ and is a measure of the degree of spatial restriction of the motion ($\mathcal{S} = 0$ when the motion is isotropic and $\mathcal{S} = 1$ if it is completely restricted). Any dynamical model of motion compatible with the experimental data should thus be such that the corresponding calculated value of $\mathcal{S}^2$, $\mathcal{S}^2_{theor}$, is compatible with its experimental value. For the lysine side-chain motions, we have selected a model of restricted diffusion about each C–C bond defined by a mean position [defined by $\alpha_{i,i+1}$ or α_{iF}, the dihedral angles between $C_{i-2}C_{i-1}$ and C_iC_{i+1} on the one hand and $C_{i-2}C_{i-1}$ and C_iH_i on the other viewed along the $C_{i-1}C_i$ bond,

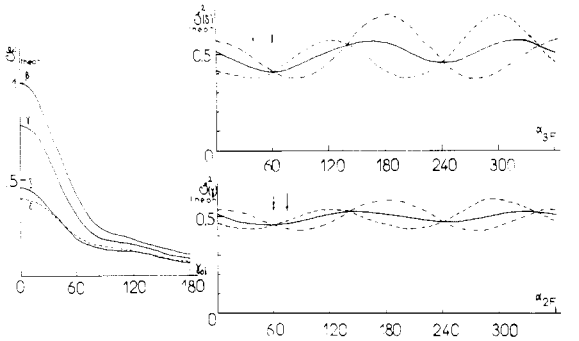

FIGURE 1: (Left) Values of the order parameters corresponding to C_β, C_γ, C_δ, and C_ϵ for an all-trans configurations and $\gamma_{01} = 30°$ (C_β), $\gamma_{01} = 45°$ (C_γ), and $\gamma_{03} = 20°$ (C_δ). (Right) Dependence of (---) $\mathcal{S}^2(C_{i1})$ [$\alpha_{iF1} = \alpha_{i,i+1} - 2\pi/3$], (- - -) $\mathcal{S}^2(C_{i2})$ [$\alpha_{iF2} = \alpha_{i,i+1} + 2\pi/3$], and (—) $\mathcal{S}^2(C_i)$ [methylene carbon] with $C_i = C_\gamma$ (bottom) and C_δ (top), as a function of α_{2Fi}; $\alpha_{1,3} = -166°$ corresponds to the crystallographic data. The values of α_{2F} and α_{3F} corresponding to the crystallized complex (arrow) and to the trans configuration (double arrow) of the side chain are also indicated.

i going from 0 (C_α) to n, the number of carbons in the side chain]. We establish in the supplementary material (see paragraph at end of paper regarding supplementary material) the following expressions of $\mathcal{S}^2_{theor}$ for each of the C_i atoms:

$$\mathcal{S}^2_{theor}(C_\beta) = \sum_a \frac{\sin^2 a\gamma_{01}}{a^2\gamma_{01}^2}[d^2_{b0}(\theta)]^2 \qquad (4)$$

$$\mathcal{S}^2_{theor}(C_\gamma) = \sum_{acc'} \frac{\sin^2 a\gamma_{01}}{a^2\gamma_{01}^2} \frac{\sin c\gamma_{02}}{c\gamma_{02}} \frac{\sin c'\gamma_{02}}{c'\gamma_{02}} d^2_{ac}(\theta)\, d^2_{ac'}(\theta)\, d^2_{c0}(\theta)\, d^2_{c'0}(\theta) \cos \alpha_{2F}(c - c') \qquad (5)$$

$$\mathcal{S}^2_{theor}(C_\delta) = \sum_{acc'dd'} \frac{\sin^2 a\gamma_{01}}{a^2\gamma_{01}^2} \frac{\sin c\gamma_{02}}{c\gamma_{02}} \frac{\sin d\gamma_{03}}{d\gamma_{03}} \frac{\sin c'\gamma_{02}}{c'\gamma_{02}} \frac{\sin d'\gamma_{03}}{d'\gamma_{03}} \cos \alpha_{23}(c' - c) \cos \alpha_{3F}(d' - d)\, d^2_{ac}(\theta)\, d^2_{cd}(\theta)\, d^2_{ac'}(\theta)\, d^2_{c'd'}(\theta)\, d^2_{d0}(\theta)\, d^2_{d'0}(\theta) \qquad (6)$$

$$\mathcal{S}^2_{theor}(C_\epsilon) = \sum_{acc'dd'ee'} \frac{\sin^2 a\gamma_{01}}{a^2\gamma_{01}^2} \frac{\sin c\gamma_{02}}{c\gamma_{02}} \frac{\sin c'\gamma_{02}}{c'\gamma_{02}} \frac{\sin d\gamma_{03}}{d\gamma_{03}} \frac{\sin d'\gamma_{03}}{d'\gamma_{03}} \frac{\sin e\gamma_{04}}{e\gamma_{04}} \frac{\sin e'\gamma_{04}}{e'\gamma_{04}} \cos \alpha_{23}(c' - c) \cos \alpha_{34}(d' - d) \cos \alpha_{4F}(e' - e)\, d^2_{bc}(\theta)\, d^2_{bc'}(\theta)\, d^2_{cd}(\theta)\, d^2_{cd'}(\theta)\, d^2_{de}(\theta)\, d^2_{d'e}(\theta)\, d^2_{e0}(\theta)\, d^2_{e'0}(\theta) \qquad (7)$$

where $\theta = 70.5°$ is the complement of the $C_{i-1}C_iH_i$ or $C_{i-1}C_iC_{i+1}$ valence angles. It should be pointed out here (cf. supplementary material) that these expressions are, as required, identical with those giving the long time limit of the dipolar autocorrelation functions given by Wittebort & Szabo (1978) for this model of motion. Figure 1 (left panel) presents the dependence of $\mathcal{S}^2_{theor}$ for the different carbon nuclei of a lysine side chain for a selected set of γ_{0i} values.

These equations thus allow estimation of γ_{0i} from the lowest energy conformation of the side chain, for example, as in our case, that found by X-ray crystallography, and from the $\mathcal{S}^2$ values obtained through T_1 measurements at two frequencies. The necessity of simultaneous fitting of γ_{0i} and τ_i to relaxation data is thus avoided.

The above theory which is established for a carbon relaxed by a single proton may be easily extended to $^{13}CH_2$ or $^{13}CH_3$ carbons if the cross-correlation effects are neglected. By use of eq 1, eq 2 and 3 are valid if T_1 is replaced by nT_1 (n = number of protons on the ^{13}C atom) and $\mathcal{S}^2$ by $\tilde{\mathcal{S}}^2$, the mean

value $\sum_{j=1}^{n} \mathcal{S}_i^2/n$. In particular, the dependence of $\tilde{\mathcal{S}}^2(C_\gamma)$, $\tilde{\mathcal{S}}^2(C_\delta)$, and $\tilde{\mathcal{S}}_2(C_\epsilon)$ on the mean conformation of the lysine side chain (see supplementary material) is smaller than that of the corresponding values for each methylene ^{13}C–H bond (see Figure 1, right panel).

The effective correlation times τ_{ei} given by eq 3 for each C_i atom may also be used theoretically to find the microscopic correlation times τ_i. In fact, we have not tried to find the exact relations between τ_i and τ_{ei}, but we have used the values of γ_{0i} found above to fit directly τ_i to the theoretical expressions of T_{1i} for multiple restricted rotation (Wittebort et al., 1980). As shown below, the good fitting obtained by this procedure entirely confirms the validity of the model-free analysis in our own experimental conditions.

Cross-Relaxation Effects. The above discussion assumes that a given carbon nucleus is relaxed by one or two uncorrelated protons. Within ^{13}C-enriched molecules, the magnetization of the various ^{13}C nuclei changes after a π pulse as a system of magnetizations coupled by cross-relaxation interactions (London et al., 1973, 1982). London et al. (1982) have already investigated cross-relaxation effects on the magnetization of individual ^{13}C nuclei of a nonselectively enriched glycine. This treatment may be generalized to molecules undergoing internal motions. We have investigated the two following species:

$$C_AO–C_MH–C_PH_3 \quad \text{(alanine)}$$

$$C_AO–C_MH–C_PH_2 \quad \text{(other amino acids)}$$

assuming a restricted diffusion motion about the C_M–C_P bond. Adjacent ^{13}C–^{13}C dipolar interactions and the interactions of a ^{13}C nucleus with protons on the adjacent carbon have been included by using the following parameters: C_iH_i = 1.09 Å; $H_PH_{P'}$ = 1.79 Å; C_MC_P = 1.53 Å; C_AC_P = 2.48 Å; C_MH_P = 2.16 Å; $C_MC_PH_P$ = 109.5°; $C_PC_MH_P$ = 28.5°. The dipolar spectral densities for such motions allow one to calculate the corresponding first-order rates f_{ij} and g_i using the relations of Solomon (1955). It is thus possible to completely define the following equations describing the time dependence of the coupled magnetizations under proton decoupling for each set of parameters (overall correlation time τ_α, internal correlation time τ_β, and angle of rotation γ_{01}):

$$\frac{dA_z}{dt} = -g_A(A_z - A_0) - f_{AM}(M_z - M_0) - f_{AP}(P_z - P_0) + \sum_i f_{AX_i}X_{0i}$$

$$\frac{dM_z}{dt} = -f_{AM}(A_z - A_0) - g_M(M_z - M_0) - f_{PM}(P_z - P_0) + \sum_i f_{MX_i}X_{0i} \qquad (8)$$

$$\frac{dP_z}{dt} = -f_{AP}(A_z - A_0) - f_{MP}(M_z - M_0) - g_P(P_z - P_0) + \sum_i f_{PX_i}X_{0i}$$

where A_0, M_0, and P_0 are the thermal equilibrium magnetizations of the ^{13}C nuclei A, M, and P and X_{0i} is that of the various protons present in the molecule. System 8 is then integrated by using standard procedures (London et al., 1982).

The main results of the calculations are the following: (1) For the small value of τ_α ($\tau_\alpha < 10^{-10}$ s) characteristic of the free peptides, cross-relaxation and relaxation by nuclei other than directly bound protons are negligible. (2) For values of τ_α characteristic of elastase–peptide complexes ($\tau_\alpha \sim 1.4 \times 10^{-8}$ s; $\tau_\beta < 10^{-10}$ s) cross-relaxation leads to significantly

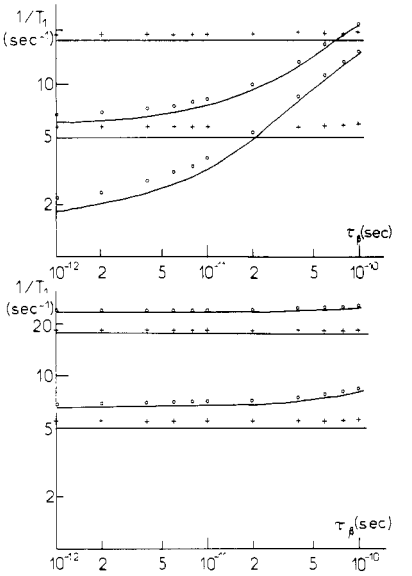

FIGURE 2: (Top) Relaxation rates at 25 (higher rate) and 50 MHz (lower rate) of the $^{13}C_\alpha H$(+) and $^{13}C_\beta H_3$ (○) nuclei, taking into account the cross-relaxation and relaxation by nuclei other than the adjacent protons. The continuous line corresponds to the same nuclei but without taking into account these effects. The parameters determining the motion are $\tau_\alpha = 1.4 \times 10^{-8}$ s and free rotation about the C_α–C_β bond. (Bottom) The same as the top panel but for a $^{13}C_\alpha H$–$^{13}C_\beta H_2$ group whose motion is determined by $\tau_\alpha = 1.4 \times 10^{-8}$ s, $\gamma_{01} = 40°$, and τ_β as variable.

nonexponential recovery curves only at the highest frequency used (125 MHz). (3) The relative contribution to the relaxation of the nuclei other than directly bound protons decreases when $1/\tau_\beta$ or γ_{01} decreases (Figure 2) as due to the geometry limited to three carbons with the peptide backbone carbons rigidly bound to the protein. Therefore (a) the contributions of the ^{13}C nuclei to the relaxation do not depend on internal motions, and (b) at 25 and 50 MHz the internal motions generally reduce the relaxation rate by adjacent protons, leading thus to a relative increase of the ^{13}C–^{13}C contribution.

In all experimental conditions used for the measurements the neglect of cross-relaxation effects and ^{13}C–^{13}C relaxation thus leads to an overestimation of the apparent ^{13}CH relaxation rate of less than 12%, which is not significantly larger than the experimental precision. In particular we were not able to measure significant differences for the relaxation rates of ^{13}C nuclei corresponding to different isotopomers (e.g., for the central component of ^{13}C–^{12}C and the external doublet ^{13}C–^{13}C of the $^{13}C_\beta$ atom of CF_3CO-[^{13}C]Ala-Ala_2).

Cross-Correlation Effects. This latter observation shows in addition that the cross-correlation dipolar densities involving two different ^{13}C nuclei are also negligible. Cross-correlation dipolar densities involving two adjacent protons are known, however, to affect the ^{13}C magnetization recovery in $^{13}CH_2$ or $^{13}CH_3$ groups of macromolecules (Werbelow & Grant, 1975a,b). The biexponential recovery curves of a $^{13}CH_3$ or $^{13}CH_2$ group after a π pulse and under proton decoupling were thus calculated, according to Werbelow & Grant (1975b). These calculations are presented in Figure 3 for a $^{13}CH_3$ (top) and a $^{13}CH_2$ (bottom) group, both in the free peptide (left) and in the complex (right), assuming free rotation around the C_α–C_β bond. Cross-correlation effects appear significant only for a methyl group within the complex. A nonlinear regression analysis of these data assuming a single exponential gives apparent relaxation rates of 7.5 and 2.1 s^{-1} at 25 and 50 MHz, respectively. These values should be compared to the corresponding values of 6.2 and 2.0 s^{-1} calculated for a methyl group when cross-correlation is neglected. Neglecting cross-corre-

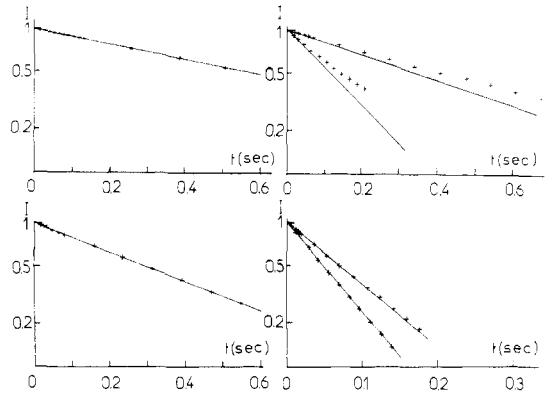

FIGURE 3: (Left) Recovery curves of a $^{13}CH_3$ group (top) and a $^{13}CH_2$ group (bottom) taking into account (+) and neglecting (—) the cross-correlation effects. (Top) $\tau_\alpha = 5.5 \times 10^{-11}$ s and $\tau_\beta = 8 \times 10^{-12}$ s; (bottom) $\tau_\alpha = 7 \times 10^{-11}$ s and $\tau_\beta = 10^{-10}$ s. The frequence of observation is 25 or 50 MHz (extreme narrowing condition). (Right) Recovery curves of $^{13}CH_3$ group (top) and a $^{13}CH_2$ group (bottom) taking into account (+) and neglecting (—) the cross-correlation effects. (Top) $\tau_\alpha = 1.4 \times 10^{-8}$ s and $\tau_\beta = 2 \times 10^{-12}$ s; (bottom) $\tau_\alpha = 1.4 \times 10^{-8}$ s and $\tau_\beta \approx 10^{-10}$ s. The slower recovery curves correspond to a ^{13}C NMR frequency of 50 MHz, the other corresponding to 25 MHz.

lation thus leads to an underestimation of the apparent ^{13}CH relaxation rates smaller than 20% at 25 MHz and much smaller at higher frequencies.

Neglecting both cross-correlation and cross-relaxation effects of nuclei other than the directly bound protons leads to some error compensation. So, the recovery curves for ^{13}C nuclei in the elastase complex at 25 or 50 MHz can be confidently analyzed according to the model-free approach of Lipari & Szabo (1982).

Effects of the Chemical Exchange between the Free and the Bound States. In solution the peptide continuously exchanges between the free, I, and bound, EI, states according to the following relation:

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI$$

In the classical inversion recovery experiment used for measuring T_1, the time evolution of the longitudinal magnetizations M_z^I and M_z^{EI} corresponding to these two states is described by the following coupled generalized Bloch equations:

$$\begin{aligned} \frac{dM_z^I}{dt} &= -\frac{M_z^I - M_0^I}{T_1^I} - k_{on}(E)M_z^I + k_{off}M_z^{EI} \\ \frac{dM_z^{EI}}{dt} &= -\frac{M_z^{EI} - M_0^{EI}}{T_1^{EI}} + k_{on}(E)M_z^I - k_{off}M_z^{EI} \end{aligned} \tag{9}$$

with the initial conditions

$$M_z^I(0) = -M_0^I \qquad M_z^{EI}(0) = -M_0^{EI}$$

M_0^I and M_0^{EI} are the thermal equilibrium magnetizations such that $M_0^I = p_I M_0$ and $M_0^{EI} = p_{EI}M_0$, with M_0 being the total thermal equilibrium magnetization of the ^{13}C nucleus under investigation and p_I and p_{EI} the proportions of free, I, and bound, EI, inhibitor. The system is integrated by using the same procedure as for the calculation of the cross-relaxation effects. $M_z^I(t)$, $M_z^{EI}(t)$, and the total magnetization $M_z(t) = M_z^I(t) + M_z^{EI}(t)$ are each a sum of two exponentials. However, for the shorter relaxation times T_1^{EI}, found in our work (corresponding to $^{13}C_\beta H_2$ of Lys at 25 MHz), for $k_{off} \sim 100$ s^{-1} (this is a lower limit for k_{off} for the two peptides under investigation), and for $p_B = 0.20$ (the largest value of p_B investigated at this frequency), the fit of the evolution of $M_z(t)$ to a single exponential is better than 1%, and the difference between the corresponding first-order relaxation rate and the theoretical value calculated from the approximated relation

$$\frac{1}{T_{1obsd}} = \frac{P_I}{T_{1I}} + \frac{P_{EI}}{T_{1EI}} \tag{10}$$

does not exceed 7%. For smaller values of p_{EI} and for the other ^{13}C nuclei, the error is smaller at 25 MHz. At 50 MHz the error is still smaller due to longer values of T_1^{EI}. We thus have analyzed the effect of exchange on the observed relaxation rates according to eq 10, corresponding in fact to fast exchange as compared to relaxation. However, the exchange in certain cases can be sufficiently slow as compared to the chemical shift to induce significant effects on the line shapes (cf. below on the Ala-containing peptide). In the experiments the rato $(E_0)/(I_0)$ has been identified with p_{EI} since the total inhibitor concentration (I_0) is always larger than the enzyme concentration (E_0) and than the dissociation constant K_I of the complex $(I_0) > 10^{-3}$ M; $K_I = 1.5 \times 10^{-5}$ M.

Relaxation of the Free Peptide. Extreme narrowing condition prevails for the peptide alone in solution, and T_1 always appears independent of the observation frequency. Strong restriction of the motions of the side chain in the free peptides will be shown furthermore to be very improbable. The model-free approach is thus not useful in this case, and we analyzed the relaxation times of the side-chain carbons according to the free diffusion model of Levine et al. (1973) assuming the C_α carbon exhibits also an overall unrestricted diffusional motion.

Materials and Methods

Synthesis of the 85% $^{13}C(U)$-Enriched Peptides. The 85% $^{13}C(U)$[1]-enriched L-amino acids were prepared biosynthetically in large scale at the Service de Biochimie, Commissariat à l'Energie Atomique, Saclay, by R. Mermet-Bouvier from hydrolysates of green algae *Spirulina Maxima* (Tran-Dinh et al., 1974).

N^α-Butyloxycarbonyl-protected amino acids were obtained from Protein Research Foundation (Japan); N^α-Boc-L-[^{13}C-(U)]alanine was prepared from 10 mmol of L-[$^{13}C(U)$]alanine with the Boc-ON[1] reagent according to Itoh et al. (1977) at a yield of 80%. N^ϵ-Z-L-[$^{13}C(U)$]lysine was prepared from 10 mmol of L-[$^{13}C(U)$]lysine according to Erickson et al. (1973) and then reacted with Boc-ON reagent. N^α-Boc-N^ϵ-Z-L-[$^{13}C(U)$]lysine was obtained as an oil at a yield of 70%.

Peptide synthesis was carried out with a Beckman 990 B synthesizer with a EEDQ (Aldrich) coupling program (Sipos & Gaston, 1974). Starting from 2.5 mmol of Boc-L-alanine/3 g of 1% cross-linked resin (Labs system), the coupling and recoupling were performed with 2 equiv of Boc-amino acid in CH_2Cl_2 for 6 h. However, for the labeled amino acids only 1 and 0.5 equiv were used for coupling and recoupling, respectively. Trifluoroacetylation on the Boc-free peptidyl-resin was performed with 3 equiv of trifluoroacetic anhydride (Weygand et al., 1957) in CH_2Cl_2 at room temperature for 2 h. The ninhydrin test according to Kaiser et al. (1970) was used to check trifluoroacetylation. After HF cleavage in the presence of anisole, the crude peptide was purified by partition chromatography on Sephadex G-25 fine (80 cm × 2.5 cm column) eluted with 1-butanol/pyridine/acetic acid 0.1% v/v 5/3/11. Fractions were checked by TLC. Amino acid analysis was as follows: (I) CF_3CO-L-[$^{13}C(U)$]Ala-L-Ala-L-Ala (150 mg; yield 50%), only Ala is found; (II) CF_3CO-L-[$^{13}C(U)$]-

[1] Abbreviations: U, uniformly; N, *p*-nitrophenyl ester; Z, carbobenzoxy; Boc, *tert*-butoxycarbonyl.

Table I: Relaxation Times T_{1EI} of the Various ^{13}C Nuclei of CF_3CO-[^{13}C]Ala-Ala_2 and CF_3CO-[^{13}C]Lys-Ala_2 in the Complex at 25 and 50 MHz[a]

peptide	^{13}C				
	C_α	C_β	C_γ	C_δ	C_ϵ
CF_3CO-[^{13}C]Ala-Ala_2 (25 MHz)	0.043 (0.005)	0.143 (0.015)			
CF_3CO-[^{13}C]Ala-Ala_2 (50 MHz)	0.181 (0.02)	0.493 (0.09)			
CF_3CO-[^{13}C]Lys-Ala_2 (25 MHz)	0.043 (0.005)	0.0356 (0.006)	0.061 (0.005)	0.054 (0.006)	0.10 (0.01)
CF_3CO-[^{13}C]Lys-Ala_2 (50 MHz)	0.207 (0.050)	0.142 (0.04)	0.177 (0.030)	0.200 (0.040)	0.260 (0.030)

[a] Values in parentheses correspond to standard deviations obtained by nonlinear regression analysis of the best estimation of relaxation times T_{1obsd} from the experimental data according to the relation $1/T_{1obsd} = p_{EI}/T_{1EI} + p_I/T_{1I}$.

Lys-L-Ala-L-Ala (120 mg; yield 40%), Lys, 1.02; Ala, 1.98.

Purification of Elastase. Porcine pancreatic elastase was purified and tested as described earlier (Dimicoli et al., 1976). The two peptides are competitive inhibitors for the hydrolysis of Succ-Ala_3-NH-C_6H_4-*p*-NO_2 by elastase. Their inhibition constants derived from Dixon plots are 1.5×10^{-5} M and 1.7×10^{-5} M, respectively, for CF_3CO-[^{13}C]Ala-Ala_2 and CF_3CO-[^{13}C]Lys-Ala_2 at 30 °C in 10^{-2} M acetate buffer, pH 5. The slightly improved affinities as compared to those reported earlier (Dimicoli et al., 1979) are due to the lower acetate concentration used in the present study, the acetate ion being itself an inhibitor of the enzyme.

NMR Experiments. The spectrum of a 85% ^{13}C-enriched residue in a peptide has been described elsewhere (Fermandjian et al., 1975). In order to obtain the apparent T_1 values of each ^{13}C-labeled nucleus, inversion recovery measurements $(180°-\tau-90°-\tau')_n$ have been performed at 25 and 50 MHz.

The time delay τ' was always larger than $4T_1$. High power noise heteronuclear ^1H spin decoupling was used systematically. The 90° pulse length was 45 and 60 μs at 25 and 50 MHz for a Varian XL 100 and a Bruker WH 200 spectrometer, respectively. The total magnetization $S(\tau)$ was recorded by integration of the corresponding multiplet signals. The extrapolation of $S(\tau)$ at $\tau = 0$ was not rigorously equal to $-S(\infty)$ but had a value going from $-0.80S(\infty)$ to $-0.95S(\infty)$ depending on the conditions. This should not affect the T_1 values which were obtained by nonlinear regression fitting with three parameters. Some spectra have been also recorded at 125 MHz on a WH 500 Bruker spectrometer. All experiments were performed in 10^{-2} M acetate buffer (uncorrected pD 5), at 30 ± 2 °C.

Results and Discussion

The ^{13}C relaxation has been measured for CF_3CO-[^{13}C]-Ala-Ala_2 and CF_3CO-[^{13}C]Lys-Ala_2 at 25 and 50 MHz. Some spectra have been recorded at higher frequency (125 MHz) which allowed us to observe the pure complex of the first inhibitor.

Effect of Complex Formation on ^{13}C Chemical Shifts. Figure 4 presents the aliphatic part of the high resolution spectrum of the free CF_3CO-[^{13}C]Ala-Ala_2 obtained under proton decoupling. When the enzyme is added to the peptide solution at the same concentration (10^{-3} M), the peptide is almost entirely bound to the enzyme ($K_I = 1.5 \times 10^{-5}$ M) and the ^{13}C resonances of the labeled Ala are still observable. The complex formation induces, however, a large upfield shift of the $^{13}C_\beta$ resonance, together with a half-height line width going at $P_{EI} = 1/3$ through a maximum of about 250 Hz which exceeds $J_{C_\alpha C_\beta}$ (34.3 Hz) (Fermandjian et al., 1975). The exchange rate between free and bound peptide is thus comparable to the chemical shift difference. The estimated upfield shift in the complex is around 1.2 ppm and the first-order dissociation constant around 300 s^{-1} in agreement with the value of 600 s^{-1} found by ^{19}F NMR for CF_3CO-Ala_3 in slightly

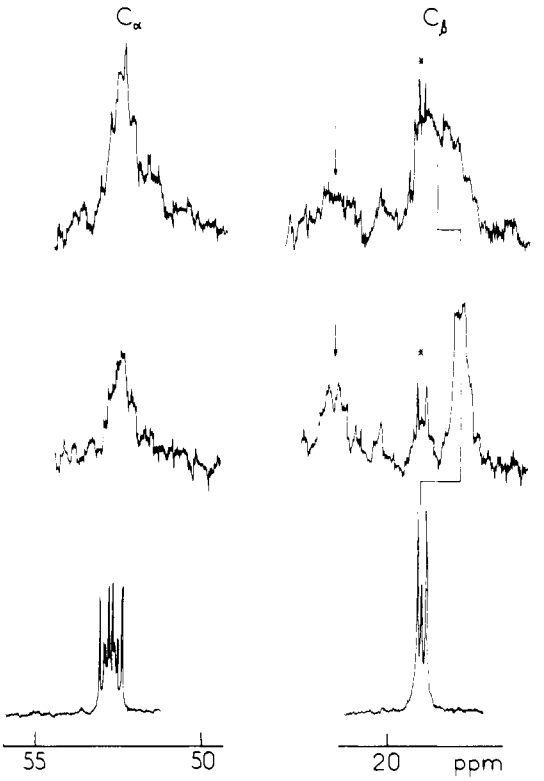

FIGURE 4: (Bottom) Spectrum of free 2×10^{-2} M CF_3CO-[^{13}C]-Ala-Ala_2 in 10^{-2} M acetate buffer, pD 5. (Middle) Spectrum of 1.1×10^{-3} M CF_3CO-[^{13}C]Ala-Ala_2 in the presence of 10^{-3} M elastase in the same buffer. (Top) Spectrum of 2.2×10^{-3} M CF_3CO-[^{13}C]Ala-Ala_2 in the presence of 10^{-3} M enzyme. The frequency of observation is 125 MHz. (Asterisk) Hydrolyzed peptide. Peaks indicated by (↓) correspond to elastase resonances.

different conditions (Dimicoli & Bieth, 1977). The $^{13}C_\alpha$ resonance is also slightly broadened in the presence of the enzyme and exhibits only a very small upfield shift (<0.2 ppm in the complex).

Complex formation of CF_3CO-[^{13}C]Lys-Ala_2 was obtained at 50 MHz with about 30% bound peptide. In this case the aliphatic part of the ^{13}C spectrum (Figure 5) exhibits neither a splitting of the resonances nor a large broadening upon complexation, and no significant chemical shift variation is observed under these conditions of fast exchange. This contrasts sharply with the alanine-containing peptide.

Spin–Lattice Relaxation Times. The magnetization recovery curves appear as single exponentials for all aliphatic carbons at both 25 and 50 MHz (Figure 6). For both peptides complex formation results in an increased observed relaxation rate proportional to the fraction p_{EI} of bound peptide (Figure 7). Under such conditions it is possible to extrapolate linearly the observed rates to $p_{EI} = 1$. The values corresponding to fully bound peptide are given in Table I. This shows that the relaxation rates for the C_α carbons of the labeled residue in both peptides bound to elastase are very close. If this carbon

Table II: Generalized Order Parameters[a] and Angles γ_{0i} Characterizing CF_3CO-[^{13}C]Ala-Ala_2 and CF_3CO-[^{13}C]Lys-Ala_2 in the Complex

peptide	C_β	C_γ	C_δ	C_ϵ	γ_{01} (deg)	γ_{02} (deg)	γ_{03} (deg)	γ_{04} (deg)
CF_3CO-[^{13}C]Ala-Ala_2	0.125				free			
	0.111				free			
CF_3CO-[^{13}C]Lys-Ala_2	0.84	0.43	0.54	0.25	25	45	0	50
	0.71 (15.48)[b]	0.38 (17.62)[b]	0.46 (19.58)[b]	0.205 (24.07)[b]	35	52	0	58

[a] Two values are given for each carbon corresponding to $\tau_\alpha = 1.4 \times 10^{-8}$ and 1×10^{-8} s, respectively. [b] In parentheses is the isotropic temperature coefficient B_{i+1} in the crystallized complex.

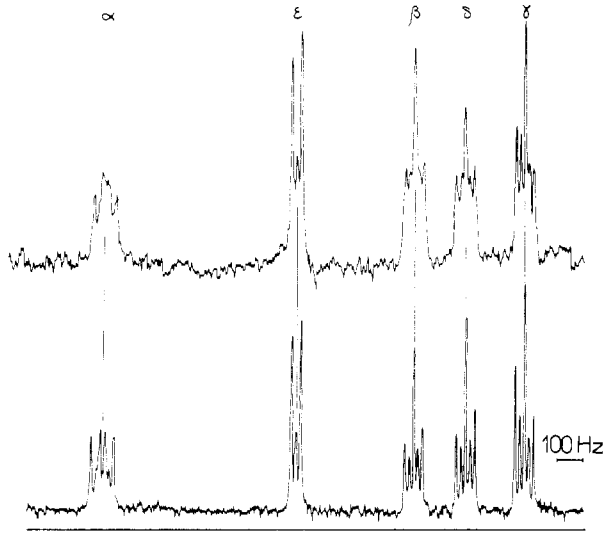

FIGURE 5: (Bottom) Spectrum of 2.15×10^{-2} M CF_3CO-[^{13}C]-Lys-Ala_2 in 0.01 M acetate buffer, pD 5. (Top) Spectrum of 4×10^{-3} M CF_3CO-[^{13}C]Lys-Ala_2 in the presence of 8.9×10^{-4} M elastase in the same buffer. The ^{13}C frequency of observation is 50 MHz. The various resonances are attributed to $^{13}C_\alpha$, $^{13}C_\epsilon$, $^{13}C_\beta$, $^{13}C_\delta$, and $^{13}C_\gamma$, respectively, going upfield according to Wittebort et al. (1980).

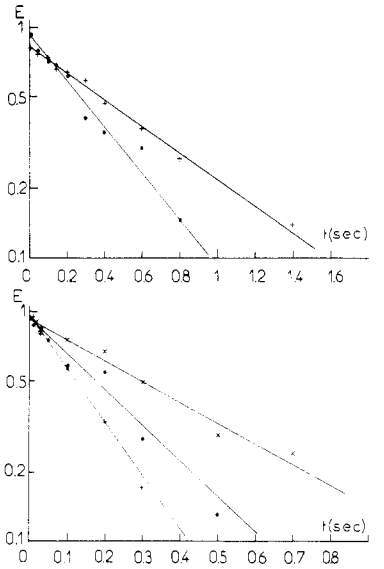

FIGURE 6: (Top) Semilogarithmic representation of $E = [S(\tau) - S(\infty)]/2S(\infty)$ as a function of τ after a π pulse for the C_α (●) and C_β (+) carbons of 4×10^{-3} M CF_3CO-[^{13}C]Ala-Ala_2 in the presence of 8.9×10^{-4} M elastase in 10^{-2} M acetate buffer, pD 5, at 50 MHz. (Bottom) Semilogarithmic plot of E as a function of τ for the C_α (●), C_β (+), and C_ϵ (×) carbons of 10^{-2} M CF_3CO-[^{13}C]Lys-Ala_2 in the presence of 10^{-3} M elastase in 10^{-2} M acetate buffer, pD 5, at 25 MHz. The straight lines are the theoretical values of E obtained by nonlinear regression analysis.

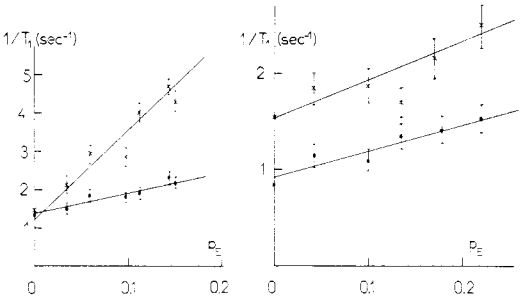

FIGURE 7: Plot of $1/T_1$ vs. p_{EI} for various ^{13}C nuclei in different experimental condition. (Left) CF_3CO-[^{13}C]Ala-Ala_2 at 25 MHz: $^{13}C_\alpha$ (+); $^{13}C_\beta$ (●). (Right) CF_3CO-[^{13}C]Lys-Ala_2 at 50 MHz: $^{13}C_\alpha$ (+); $^{13}C_\epsilon$ (●).

itself is tightly bound to the enzyme, the corresponding correlation time for the overall tumbling of the protein must be between 10^{-8} and 1.4×10^{-8} s, which is in agreement with the size of this protein (Yguerabide et al., 1970).

The internal motion of the alanine $^{13}C_\beta H_3$ group is certainly free and rapid since $T_1(C_\beta)/T_1(C_\alpha)$ is 3.33 and 2.72 at 25 and 50 MHz, respectively, which is very near the theoretical value of 3 predicted from the Woessner model (Woessner, 1962). In the case of the side chain of lysine, the above model does not hold. Restriction of the motion about each C–C bond thus has to be taken into account. We use the procedure described under Theory to characterize these motions.

Order Parameters and Amplitude of the Side-Chain Motions. We have shown under Theory that the single exponential magnetization recovery curves found in our experiments may be used directly to find the order parameters $\mathcal{S}^2$ of each ^{13}C atom using eq 2. In Table II we report the estimations of $\mathcal{S}^2$ for the two peptides bound to elastase and corresponding to the two limiting values of the overall correlation time τ_α compatible with the relaxation time of the C_α carbon, i.e., 10^{-8} s ($T_1^R = 0.0465$ s and $\tilde{T}_1^R = 0.150$ s) and 1.4×10^{-8} s ($T_1^R = 0.057$ s and $\tilde{T}_1^R = 0.202$ s). As already found by Wittebort & Szabo (1978), the estimations of $\mathcal{S}^2$ depend only moderately on the choice of τ_α.

As a consequence the $\mathcal{S}^2$ values of 0.111–0.126 for the CF_3CO-[^{13}C]Ala-Ala_2 labeled methyl group are not significantly different from the theoretical value of 0.111 expected from a freely rotating methyl group, in agreement with the above remark on the internal motion of this group. This further confirms that the C_α carbon of the first residue, Ala or Lys, is rigidly bound to the protein since wobbling (Richarz et al., 1980) of the peptide backbone would lead to smaller values of $\mathcal{S}^2(C_\beta)$ parameters if free rotation of the methyl group still occurs.

On the other hand, the high values of $\mathcal{S}^2$ found for the carbon atoms of [^{13}C]Lys are analyzed by using eq 4–7 and assuming a lowest energy trans conformation ($\alpha_{23} = \alpha_{34} = \alpha_{45} = 180°$; $\alpha_{2F} = \alpha_{3F} = \alpha_{4F} = \pm 60°$) as found in the crystal. The low effect of τ_α on $\mathcal{S}^2$ entails a rather good precision on the degree of restriction of the side-chain motions, the errors on γ_{0i} never exceeding 10°. The angles γ_{0i} may be also correlated to the isotropic temperature coefficient B deduced from X-ray crystallography. It should be pointed out here that since the order parameter of carbon C_i results from the motions of the

Table III: Fitting of the Parameter τ_i to the Experimental Relaxation Data Given the γ_{0i} Values of Table II and $\tau_\alpha = 1.4 \times 10^{-8}$ s

	CF_3CO-[^{13}C]Ala-Ala_2	CF_3CO-[^{13}C]Lys-Ala_2			
	C_β	C_β	C_γ	C_δ	C_ϵ[b]
γ_{0i}	free	25	45	<20	free
τ_i	2×10^{-12}	10^{-10}	8×10^{-11}	6×10^{-11}	6×10^{-11}
T_1 (25 MHz)[a]	0.163 (0.143 ± 0.015)	0.036 (0.036 ± 0.006)	0.065 (0.061 ± 0.005)	0.06 (0.054 ± 0.006)	0.16 (0.10 ± 0.01)
T_1 (50 MHz)[a]	0.50 (0.49 ± 0.09)	0.125 (0.142 ± 0.04)	0.16 (0.178 ± 0.03)	0.16 (0.2 ± 0.04)	0.24 (0.26 ± 0.03)

[a] In parentheses following the theoretical values of T_1 are reported the corresponding experimental values. [b] The calculations have been performed with free rotation assumed for γ_{04}, showing the lack of consistency between theoretical and experimental results in agreement with $\mathcal{S}^2(C_\epsilon) = 0.25$.

Table IV: Relaxation Times T_{1I} of the Various ^{13}C Nuclei of CF_3CO-[^{13}C]Ala-Ala_2 and CF_3CO-[^{13}C]Lys-Ala_2 Alone in Solution

	^{13}C[a]				
peptide	C_α	C_β	C_γ	C_δ	C_ϵ
CF_3CO-[^{13}C]Ala-Ala_2 (25 MHz)	0.805 (0.050)	0.745 (0.04)			
CF_3CO-[^{13}C]Ala-Ala_2 (50 MHz)	0.920 (0.09)	0.780 (0.03)			
CF_3CO-[^{13}C]Lys-Ala_2 (25 MHz)	0.640 (0.06)	0.41 (0.01)	0.500 (0.05)	0.88 (0.11)	0.94 (0.10)
CF_3CO-[^{13}C]Lys-Ala_2 (50 MHz)	0.630 (0.07)	0.385 (0.05)	0.550 (0.04)	0.79 (0.09)	1.10 (0.08)

[a] Values in parentheses correspond to standard deviations obtained by nonlinear regression analysis.

Table V: Correlation Times Characterizing the Motions of the ^{13}C Nuclei of the Free CF_3CO-[^{13}C]Lys-Ala_2 and CF_3CO-[^{13}C]Ala-Ala_2 Peptides Assuming Free Diffusional Rotation

	rotation				
peptide	τ_α (s)	τ_β (s)	τ_γ (s)	τ_δ (s)	τ_ϵ (s)
CF_3CO-[^{13}C]Ala-Ala_2	5.5×10^{-11}	8×10^{-12}			
CF_3CO-[^{13}C]Lys-Ala_2	7×10^{-11}	10^{-10}	9×10^{-11}	2.5×10^{-11}	2.5×10^{-11}

C_i–H bonds, it has to be correlated to the isotropic temperature coefficient B_{i+1} of C_{i+1} rather than B_i of C_i (cf. also Lipari et al., 1982). It is gratifying to emphasize the simultaneous strong increase of B_{i+1} and γ_{0i} observed for the C_ϵ atom, suggesting that immobilization of the side chain is not a consequence of a strong interaction of the amino group with some residue of the enzyme but rather to contacts between the enzyme and the C_β, C_γ, or C_δ methylene groups. In fact, X-ray data show a strong contact between C_δ and the $C_{\gamma 1}H_3$ group of Val-99. Our NMR results thus suggest that such a contact still exists in solution.

Fitting of the Correlation Times τ_i to the Relaxation Data. Effective correlation times τ_{ei} may be theoretically obtained from T_1 and $\tilde{T}_1$ for each ^{13}C nucleus by using eq 3. But first the error on T_1^R and second the small values of γ_{0i} lead to very imprecise estimations of τ_{ei}. Furthermore τ_{ei} does not have the simple meaning of the correlation time τ_i. We thus preferred to use the amplitudes of motion γ_{0i} to directly fit the various parameters τ_i to the experimental relaxation times by using the complete equations giving T_1 (Wittebort & Szabo, 1978; Wittebort et al., 1980).

The best fitting for carbons C_β to C_δ was obtained for $\tau_\alpha = 1.4 \times 10^{-8}$ s, and the corresponding results are given in Table III. Somewhat less good fitting is obtained for $\tau_\alpha = 10^{-8}$ s. Furthermore, values of τ_i smaller than those reported in Table III also give good fitting to the data. This confirms the small sensitivity of T_1 to τ_i when γ_{0i} is small. Furthermore, $\omega\tau_i$ being in every case smaller than 0.03, the calculations of $\mathcal{S}^2$ from eq 2 are valid (Wittebort & Szabo, 1978). No fitting was obtained for C_ϵ assuming free rotation about the C_δ–C_ϵ bond. This was expected, considering the small value of γ_{0i} (<60°). This analysis confirms the validity of the model-free approach for obtaining $\mathcal{S}^2$ from the relaxation times.

Case of the Free Peptides. Extreme narrowing prevails for the ^{13}C NMR spectra of the free peptides (Table IV), and the measured ^{13}C relaxation rates are not sufficient to describe unambiguously their internal motions in terms of restricted diffusion. The rotation of the alanine methyl groups in CF_3CO-[^{13}C]Ala-Ala_2 is very likely free, as in the complex.

The conformation and mobility of the lysine side chain in CF_3CO-[^{13}C]Lys-Ala_2 are better analyzed from the 500-MHz ^{1}H spectrum. In the methylene groups, vicinal proton–proton coupling constants have values lying between 7.3 and 7.9 Hz, and there is no indication of significant nonequivalence of chemical shifts (the largest nonequivalence of 0.062 ppm is observed for the $C_\beta H_2$ protons). These results suggest that a practically free rotation occurs in the side chain and that no particular conformation is favored. Yet, analyzed in terms of rotamers, the results indicate that the three rotamers are almost equally populated. Since generally in residues with a $C_\beta H_2$ in the side chains the trans conformation is found less populated than the two others (Bystrov, 1976), it seems likely that some interactions in the lysine side chain lead to an increase in this rotamer in the free peptide. It is interesting to recall here that in the complex it is precisely this rotamer that is favored.

The analysis of the relaxation data in terms of free diffusion yields correlation times (Table V) that can be compared to those in the complex. In contrast with its effect on the amplitude of the side-chain motions, complexation does not lead to a strong decrease of their frequencies. In the alanine-containing peptide some increase of the rate is even possible.

Conclusion

The neglect of cross-relaxation and cross-correlation effects for the interpretation of the ^{13}C relaxation data in the enriched peptides has been shown to introduce errors in the correlation times which should be at most 30% and probably lower at 25 and 50 MHz. The difference observed for the two investigated peptide–elastase complexes is thus highly significant. The free rotation of the C_β methyl group of the labeled Ala (it appears even with a shorter correlation time of 2×10^{-12} s in the complex compared to 8×10^{-12} s in the free peptide) contrasts with the strongly restricted motion of the corresponding lysine

side chain. This difference between the two side chains was already revealed by the large upfield shift which was observed uniquely in the case of the alanine C_β carbon.

On the contrary, there is almost no difference in affinity for elastase for the two trifluoroacetylated peptides at pD 5. This should result from, at least, two opposite effects. Weak backbone interactions could be compensated by stronger side-chain interactions for the lysine-containing peptide. The very small value of γ_{03} observed for the C_δ carbon fits rather well the good contact observed between this methylene group and the $C_{\gamma 1}H_3$ methyl group of Val-99 in the crystal. Furthermore, the probably higher free energy of interaction due to favorable contacts between protein and the immobilized side chain of lysine may be compensated by unfavorable entropic effects. In any case thermodynamic investigations are necessary to confirm the last hypothesis.

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Supplementary Material Available

Derivation of δ^2_{theor} used in eq 4–7 (3 pages). Ordering information is given on any current masthead page.

Registry No. CF_3CO-L-[^{13}C(U)]Ala-L-Ala-L-Ala, 90269-14-0; CF_3CO-L-[^{13}C(U)]Lys-L-Ala-L-Ala, 90269-15-1; N^α-Boc-L-[^{13}C(U)]alanine, 72634-72-1; N^α-Boc-N^ϵ-Z-L-[^{13}C(U)]lysine, 90269-16-2.

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