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OPTICAL MEASUREMENT OF A SOLVENT-INDUCED ISOMERIZATION IN A PROLINE-CONTAINING HEXAPEPTIDE

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SUMMARY : The hexapeptide Gly-Gly-Pro-Tyr-Gly-Gly has been synthesized and its tyrosine residue converted to nitrotyrosine by reaction with tetranitromethane. When diluted from dimethylsulfoxide into aqueous solution, the nitrated hexapeptide undergoes a slow conformational change characterized by a change in the ionization state of the nitrotyrosine group. This slow reaction is not observed with peptides containing nitrotyrosine and no proline. Also, the rate and activation enthalpy of this slow conformational change suggest that it could be due to proline *cis-trans* isomerization. The possibility of measuring the rate of *cis-trans* isomerization of proline residues in a polypeptide chain is discussed.

INTRODUCTION

The pK of an ionizable group in a molecule depends on the particular environment of this group, and therefore pK changes could in principle be used to monitor conformational changes within this molecule. It has been shown previously that indeed the pK of an ionizable reporter group can provide a sensitive index of various conformational changes in a protein (1). In the present work, the tyrosine residue of the hexapeptide Gly-Gly-Pro-Tyr-Gly-Gly has been converted into 3-nitrotyrosine, the ionization of which can be easily measured by spectrophotometry (2). It is found that a rapid transfer of the nitrated peptide from Me_2SO[1] into aqueous solution results in a slow isomerization, as evidenced by the pK changes of the nitrotyrosine residue. The kinetic properties of this slow isomerization suggest that it could be the *cis-trans* isomerization of the Gly-Pro peptide bond.

MATERIALS AND METHODS : The peptides Ala-Tyr and Val-Tyr-Val were obtained from Sigma, and the hexapeptide Gly-Gly-Pro-Tyr-Gly-Gly was synthesized by the repetitive excess mixed anhydride method (3). These peptides were nitrated by tetranitromethane in the following conditions : about 10^{-3} M peptide, 8.3×10^{-2} M tetranitromethane, 10% (v/v) EtOH, in 0.5 M Tris buffer at pH 8.5. After 3 to 4 hours, the nitrated peptides were separated from

[1] Me_2SO : *dimethylsulfoxide*

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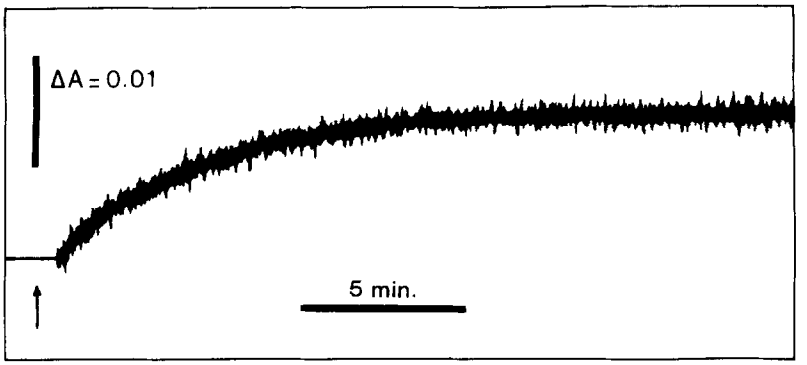

Figure 1. Recordings of the change in absorbance at 428 nm of the peptide Gly-Gly-Pro-(NO_2Tyr)-Gly-Gly upon a jump from 100% Me_2SO to 10% Me_2SO, 0.2 M cacodylate buffer, pH 6.5 at 20°. Peptide concentration is about 10^{-4} M. The arrow indicates the zero time of the kinetics.

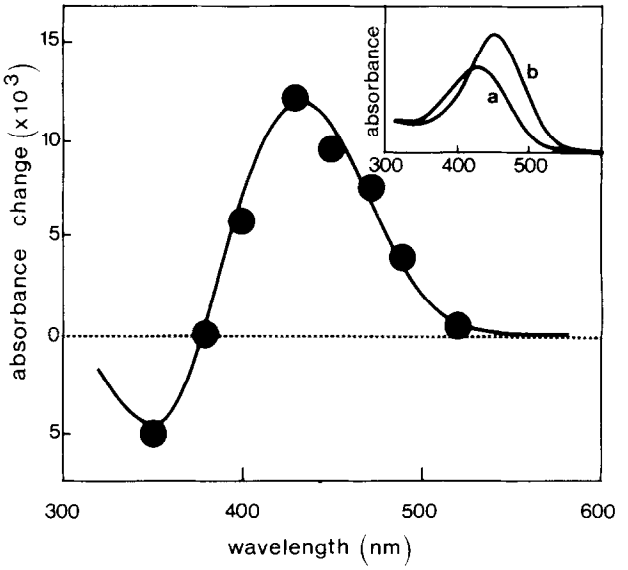

Figure 2. Dependence on the wavelength of the amplitude of the absorbance change observed upon a jump from 100% Me_2SO to 10% Me_2SO, 0.2 M cacodylate buffer, pH 6.5, at 20°. Peptide concentration is about 10^{-4}M. The solid line corresponds to the difference spectrum between the ionized and protonated forms of the peptide recorded at equilibrium.

Inset : Absorption spectra of the ionized form of the peptide in *(a)* 0.2 M cacodylate buffer and *(b)* Me_2SO.

the excess of reagent by two gel filtrations on Sephadex G-10, and then lyophilized. Amino acid analysis showed that in each case nitration has been essentially complete. Spectrophotometric measurements were performed in a Cary 17 instrument. The amplitudes of the optical changes in kinetic experiments were determined by extrapolation to zero time using first-order plots of the observed changes.

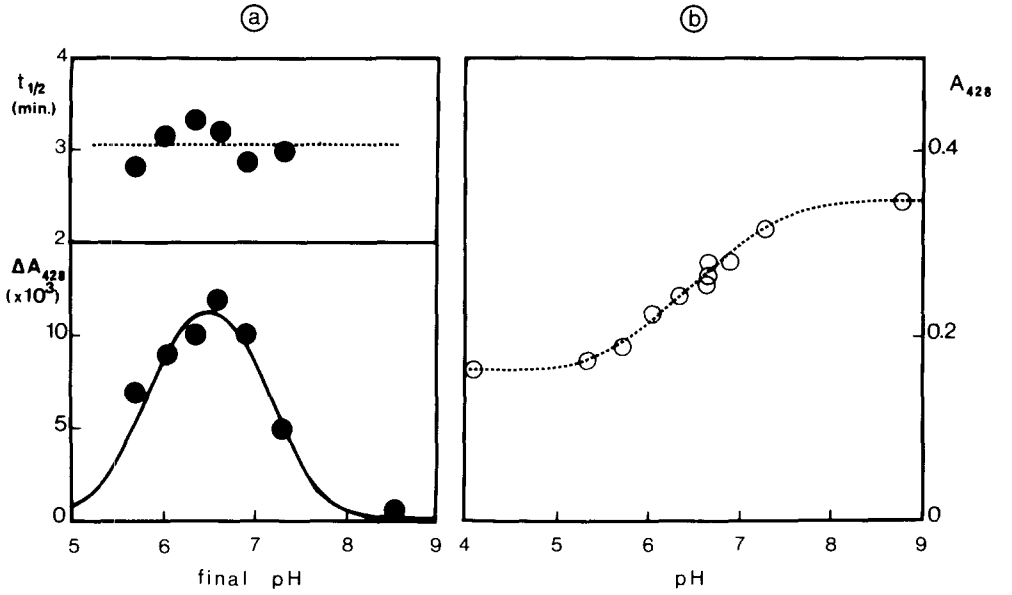

Figure 3. *(a)* Dependence on the final pH of the amplitude and half-life of the change in A_{428nm} observed upon a jump from 100% Me_2SO to 10% Me_2SO, 0.2 M cacodylate buffer, at 20°.

(b) pH titration of the nitrated peptide in 10% Me_2SO, 0.2 M cacodylate buffer, at 20°. The dashed line corresponds to the ionization curve of one group of pK 6.5. Peptide is 10^{-4}M.

RESULTS

The nitrated hexapeptide was dissolved in pure Me_2SO, and then diluted 5 to 50 times into 0.2 M cacodylate buffer at various pH values. Figure 1 shows that this rapid change in solvent results in a slow reaction, as observed at 428 nm through the absorption of the ionized form of the nitrotyrosine group. These changes in A_{428nm} follow first-order kinetics.

The absorption spectra of the nitrated hexapeptide are quite different in Me_2SO and in aqueous solution (Inset of figure 2). The wavelength dependence of the absorbance change measured upon such a "solvent-jump" does not correspond to a solvent-induced difference spectrum, Me_2SO *vs* aqueous solution; instead, it follows the difference spectrum between the protonated and ionized forms of the nitrated hexapeptide in aqueous solution (Figure 2).

The amplitude of the change in A_{428nm} observed upon such "solvent-jumps" also depends on the final pH (Figure 3). This change can be detected only between pH 5.5 and 7.5, *i.e.* in the pH range where the nitrotyrosine residue ionizes (Figure 3), and it reaches a maximum value for a final pH value of 6.5, *i.e.* at the pK of this residue. Since the final pH is strongly buffered

TABLE 1

Half-life of the reaction observed upon a jump from 100% Me_2SO to various final conditions, in 0.2 M cacodylate buffer, at pH 6.5, 25°

Final Me_2SO (%)	Wavelength (nm)	$t_{1/2}$ (sec)
2	428	135
5	428	125
10	428	135
10	300	130
20	428	140

by 0.2 M cacodylate buffer, the changes in A_{428nm} correspond to a change in the ionization state of the nitrotyrosine group. The bell-shaped curve of figure 3 is therefore a differential titration curve of the nitrated peptide between the zero and infinite times of the reaction caused by the "solvent-jump"; the zero time is that just after dilution and after completion of all rapid reactions like proton binding or dissociation. Then both the wavelength dependence of the absorbance changes (Figure 2) and the pH dependence of the changes in A_{428nm} (Figure 3) suggest that these changes are due to a change in the pK of the nitrotyrosine group during the reaction triggered by a "solvent-jump". The pK of this group measured in aqueous solution just after the dilution from Me_2SO is different from that measured in aqueous solution at equilibrium.

In contrast with its amplitude, the half-life of the reaction observed at 428 nm upon a "solvent-jump" does not depend markedly on the final pH (Figure 3). It does not depend significantly on the final amount of Me_2SO (Table 1), although it was observed that the amplitude of the changes in A_{428nm} slightly increases when decreasing the final amount of Me_2SO. When determined at a final pH of 6.5, the rate of the reaction caused by the "solvent-jump" strongly depends on temperature. The activation enthalpy $\Delta H^{\neq}$, obtained from the Arrhenius plot given in figure 4, is 18 ± 3 kcal/mole. At 300 nm, where the absorbance changes due to the ionization of the nitrotyrosine residue have the opposite sign of those at 428 nm, the same rate is measured (Table 1), which indicates that the experimental procedure is free from temperature artefacts (4).

The same experiments were repeated with two other peptides, Ala-Tyr and Val-Tyr-Val, after conversion of the tyrosine residue into nitrotyrosine.

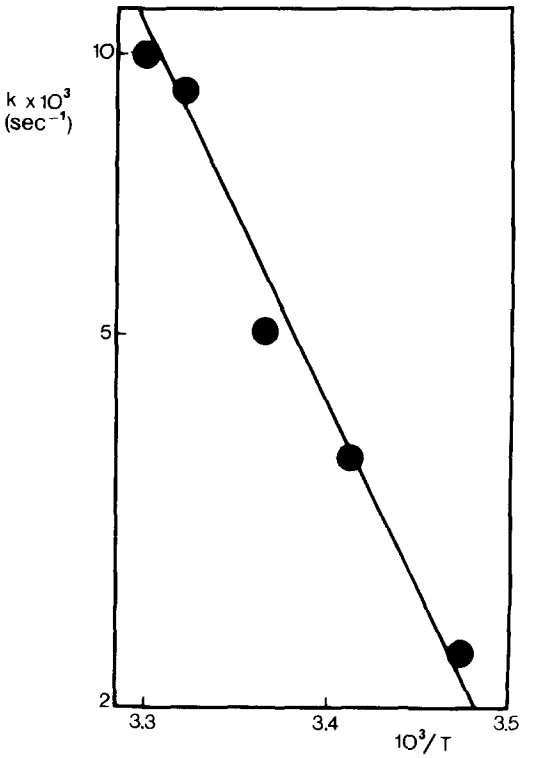

Figure 4. Arrhenius plot of the reaction observed at 428 nm upon a jump from 100% Me_2SO to 10% Me_2SO, 0.2 M cacodylate buffer, pH 6.5

In these peptides, the nitrotyrosine group has ionization and optical properties very close to those in the hexapeptide, but no change in absorbance could be detected upon a "solvent-jump".

D I S C U S S I O N

Upon changing the solvent from Me_2SO to aqueous solution, the nitrotyrosine group of the hexapeptide shows a slow change in its ionization state, as measured by absorbance changes (Figure 1). All reactions like free rotations around single bonds, proton binding or dissociation, changes in solvatation, formation or breaking of hybrogen bonds, etc..., which are expected to take place in an oligopeptide upon a solvent change, are fast and should be completed within the dead-time of the dilution. It is then likely that the slow change in ionization observed upon a "solvent-jump" is not directly due to a solvent effect, but instead is the consequence of a conformational change of the nitrated hexapeptide. The conformation of this peptide is probably different in Me_2SO and in aqueous solution. Just after

the dilution, the peptide is in aqueous solution but still with the same conformation as in Me_2SO; then it undergoes a conformational change to slowly reach the conformation that it normally has in aqueous solution. Indeed, the absorbance changes observed during this slow reaction correspond to changes in the ionization state of the nitrotyrosine group occurring in aqueous solution (Figures 2 and 3). At a constant final pH, fixed by strongly buffered conditions (0.2 M cacodylate buffer), changes in the ionization state are due to pK changes. Therefore, the pK of the nitrotyrosine group in the peptide just after the dilution, *i.e.* in aqueous solution but in the conformation that the peptide has in Me_2SO, is different from the pK in the peptide at equilibrium in aqueous solution. It is the difference between these two pK's, both corresponding to aqueous solution, which allows the detection of this conformational change.

Several arguments can be presented suggesting that the conformational change occurring upon a "solvent-jump" in the nitrated hexapeptide corresponds to the *cis-trans* isomerization of the Gly-Pro peptide bond.

1°) Such a slow conformation change is not observed with peptides containing a nitrotyrosine residue and no proline residue. This result also shows that direct solvent effects are not the cause of the slow reaction of the hexapeptide.

2°) The *cis-trans* equilibrium of a X-Pro peptide bond depends on the solvent, and most of the studied compounds show that the relative amount of the *cis* and *trans* forms is different in Me_2SO and in aqueous solution (5, 6).

3°) For oligopeptides, the *cis-trans* isomerization of a proline residue is the only reaction reported to be slow enough to agree with the time range corresponding to this conformational change.

4°) This conformational change has an activation enthalpy $\Delta H^{\neq}$ of 18 ± 3 kcal/mole, which is comparable to that measured for proline *cis-trans* isomerization (7-9).

This slow conformational change is probably due to a change in the relative amounts of the *cis* and *trans* forms of the Gly-Pro peptide bond. The pK change of the nitrotyrosine group during this reaction would arise from a pK difference for this group between the *cis* and *trans* isomers of the neighbouring proline. Indeed, the pK of ionizable groups close to a proline residue has already been shown to be different in the *cis* and *trans*

forms (5, 6, 10-12), and this seems to be true for nitrotyrosine in the hexapeptide. Then, through its pK changes, an ionizable reporter group like nitrotyrosine can measure the rate of proline *cis-trans* isomerization, although it is not involved in the isomerizing bond. Such a rate measurement could be extended to any X-Pro peptide bond by introducing an ionizable reporter group close enough and following its ionization changes. In particular, this could be of interest in the case of a polypeptide chain where the rate of proline *cis-trans* isomerization is difficult to obtain and is thought of as a critical factor for the rate of protein folding (7, 13). Preliminary experiments performed with ribonuclease support the validity of this method.

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REFERENCES :

1. Garel, J.R. (1976) Eur. J. Biochem., 70, 179-189.
2. Sokolovsky, M., Riordan, J.F., and Vallee, B.L.(1966) Biochemistry, 5, 3582-3589.
3. Beyerman, H.C. (1972) In : Chemistry and Biology of peptides, (J. Meienhofer, ed.), Ann Arbor Publishers, Ann Arbor, Mich., pp. 351-357.
4. Garel, J.R., and Baldwin, R.L. (1975) J. Mol. Biol., 94, 621-632.
5. Grathwohl, C., and Wüthrich, K. (1976) Biopolymers, 15, 2025-2041.
6. Grathwohl, C., and Wüthrich, K. (1976) Biopolymers, 15, 2043-2057.
7. Brandts, J.F., Halvorson, H.R., and Brennan, M. (1975) Biochemistry, 14, 4953-4963.
8. Cheng, H.N., and Bovey, F.A. (1977) Biopolymers, 16, 1465-1472.
9. Nall, B.T., Garel, J.R., and Baldwin, R.L. (1978) J. Mol. Biol., 118, 317-330.
10. Wüthrich, K., and Grathwohl, C. (1974) FEBS Letters, 43, 337-340.
11. Evans, C.A., and Rabenstein, D.L. (1974) J. Amer. Chem. Soc., 96, 7312-7317.
12. Fermandjian, S., Tran-Dinh, S., Savrda, J., Sala, E., Mermet-Bouvier, R., Bricas, E., and Fromageot, P. (1975) Biochim. Biophys. Acta, 399, 313-338.
13. Schmid, F.X., and Baldwin, R.L. (1978) Proc. Natl. Acad. Sci. U.S., 75, 4764-4768.