Huestis, W. H., and Raftery, M. A. (1972), *Biochem. Biophys. Res. Commun. 48,* 678.
Hunter, T., and Munro, A. (1969), *Nature (London) 223,* 1270.
Joels, N., and Pugh, L. G. C. E. (1958), *J. Physiol. 142,* 63.
Lindstrom, T. R. Noren, J. B. E., Charache, S., Lehmann, H., and Ho, C. (1972), *Biochemistry 11,* 1677.
Mansouri, A., and Winterhalter, K. H. (1973), *Biochemistry 12,* 4946.
Matwiyoff, N. A., and Needham, T. E. (1972), *Biochem. Biophys. Res. Commun. 49,* 1158.
Matwiyoff, N. A., Vergamini, P. J., Needham, T. E., Gregg, C. T., Volpe, J. A., and Caughey, W. S. (1973), *J. Amer. Chem. Soc. 95,* 4429.
Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88.
Moon, R. B., and Richards, J. H. (1972), *J. Amer. Chem. Soc. 94,* 5093.
Muirhead, H., Cox, J. M., Mazzerella, L., and Perutz, M. F. (1967), *J. Mol. Biol. 28,* 117.
Muirhead, H., and Greer, J. (1970), *Nature (London) 228,* 516.
Ogata, R. T., and McConnell, H. M. (1972a), *Proc. Nat. Acad. Sci. U. S. 69,* 335.
Ogata, R. T., and McConnell, H. M. (1972b), *Biochemistry 11,* 4792.
Ogawa, S., and Shulman, R. G. (1971), *Biochem. Biophys. Res. Commun. 42,* 9.
Olson, J. S., Anderson, M. E., and Gibson, Q. H. (1971), *J. Biol. Chem. 246,* 5919.
Olson, J. S., and Gibson, Q. H. (1972), *J. Biol. Chem. 247,* 1713.
Olson, J. S., and Gibson, Q. H. (1973), *J. Biol. Chem. 248,* 1616.
Perutz, M. F. (1969), *Proc. Roy. Soc., Ser. B 173,* 113.
Perutz, M. F. (1970), *Nature (London) 228,* 726.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219,* 131.
Schroeder, W. A., Shelton, J. R., Shelton, J. B., Cormick, J., and Jones, R. T. (1963), *Biochemistry 2,* 992.
Vergamini, P. J., Matwiyoff, N. A., Wohl, R. C., and Bradley, T. (1973), *Biochem. Biophys. Res. Commun. 55,* 453.
Yip, Y. K., Waks, M., and Beychok, S. (1972), *J. Biol. Chem. 247,* 7237.

# Carbon-13 Nuclear Magnetic Resonance Studies of Structure and Function in Thyrotropin-Releasing Factor. Determination of the Tautomeric Form of Histidine and Relationship to Biology Activity†

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ABSTRACT: The pH dependence of the $^{13}C$ chemical shifts of histidine in thyrotropin releasing factor (TRF) <Glu-His-Pro-$NH_2$ demonstrates that the $N^{\tau}$-H tautomer is the predominant form of the imidazole ring in histidine in basic solution. Protonation of the imidazole ring of His in TRF causes changes in the chemical shifts of the His residue itself as well as in the chemical shifts of the $\beta$ carbon of <Glu and in the $\beta$ and $\delta$ carbons of Pro, possibly as a result of slight changes in steric constraints on the peptide. Spin-lattice relaxation times ($T_1$) of the carbons in TRF are not affected by protonation of the imidazole ring of His. This implies that there is no large change in the rate of overall molecular reorientation nor in the relative rates of reorientation of the individual residues, hence no measurable change in conformation upon protonation of the imidazole ring of His. The $^{13}C$ chemical shifts of the highly biologically active $N^{\tau}$-methylimidazole TRF are more similar to TRF than those of the almost inactive $N^{\pi}$-methylimidazole derivative. Protonation of the *N*-methylimidazole derivatives causes chemical shift changes in the spectra of these compounds and results in spectra which resemble the protonated form of TRF. The chemical shift changes which occur upon protonation of histidine in TRF, the $N^{\pi}$- and the $N^{\tau}$-methylhistidine TRF derivatives, are of similar magnitude to the changes found in histidine monomer, $N^{\pi}$- and $N^{\tau}$-methylhistidine, respectively. The $pK_a$ values of His and *N*-methylhistidine in the above peptides reflect the $pK_a$ values of free His and the *N*-methylhistidine monomers. The $N^{\tau}$ tautomer of His in TRF is postulated to be that which interacts with the receptor. The interaction at the receptor would be a nonbonded, van der Waals type. This would explain the higher activity of the $N^{\tau}$-methylimidazole TRF.

Thyrotropin-releasing factor (TRF)[1] controls the secretion of thyrotropic hormone from the anterior pituitary gland (Schally *et al.*, 1968). The sequence of TRF is <Glu-His-Pro-$NH_2$[2] (Figure 1) (Folkers *et al.*, 1969; Burgus *et al.*, (1969a). Structure-function studies on TRF have been carried out *via* two

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[1] Abbreviation used is: TRF, thyrotropin-releasing factor.

[2] We use the convention that $N^{\pi}$ in histidine is the N atom closest to the point of attachment of the imidazole ring to the $\beta$ carbon. This is in accordance with the recently suggested IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry 11,* 1726 (1972)). All amino acids are of the L configuration.

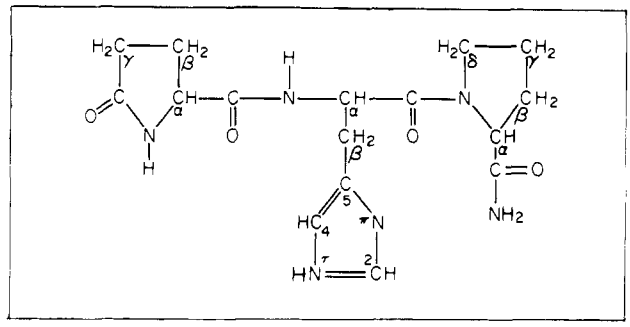

FIGURE 1: Primary sequence of TRF showing preferred tautomer and numbering scheme for imidazole.

approaches: those which investigate the types of residues necessary for hormonal activity (Burgus *et al.*, 1969b; Bowers *et al.*, 1970; Enzmann *et al.*, 1971; Chang *et al.*, 1971; Monahan *et al.*, 1972) and those which seek to determine the three-dimensional conformation of the hormone which may be effective at the receptor. The latter studies have been undertaken by minimum energy calculations (Belle *et al.*, 1972; Blagdon *et al.*, 1973; George and Kier, 1973; Burgess *et al.*, 1973), $^1$H nuclear magnetic resonance (nmr) (Fermandjian *et al.*, 1972; Boilot, 1972), $^{13}$C nmr (Deslauriers *et al.*, 1973a; Smith *et al.*, 1973), Raman spectroscopy (Bellocq *et al.*, 1973), and potentiometric titrations (Grant *et al.*, 1972).

In the present study we have determined the tautomeric form of the imidazole ring of the histidine residue in TRF. We have examined the $^{13}$C spectra of $N^{\pi}$- and $N^{\tau}$-methylimidazole TRF and found that the spectrum of the more biologically active $N^{\tau}$-methylimidazole TRF is more similar to that of the natural hormone than is that of the almost inactive $N^{\pi}$-methylimidazole TRF. Protonation of the His residue in TRF produces changes in the spectra of the <Glu and Pro-$NH_2$ residues which may be a consequence of slight conformational changes in the hormone. We conclude that the $N^{\tau}$-H tautomer of His in TRF is that which interacts with the receptor, and this hypothesis is extended to explain the hyperactivity of the $N^{\tau}$-methylimidazole TRF.

## Experimental Section

*Materials.* Thyrotropin-releasing factor (TRF) (<Glu-His-Pro-$NH_2$) was purchased from Bachem Fine Chemicals, Marina del Rey, Calif. $N^{\pi}$-Methylimidazole TRF and $N^{\tau}$-methylimidazole TRF were prepared following the procedure of Rivier *et al.* (1972). The pH meter readings ("pH"; pD = "pH" + 0.4) (Glascoe and Long, 1960) of samples were adjusted using $CD_3COOH$ or dilute solutions of $NH_4OH$ in $D_2O$.

*Methods.* $^{13}$C nuclear magnetic resonance spectra were obtained on a Varian XL-100-15 spectrometer in the Fourier transform mode, with complete proton decoupling, using a Varian 620L computer with 16K memory. Spin-lattice relaxation time ($T_1$) measurements were performed by the inversion-recovery method as described by Freeman and Hill (1970) using a pulse sequence (180°-$\tau$-90°-$T_\infty$. . . .) where $\tau$ is a variable delay time and $T_\infty$ is at least five times longer than the longest $T_1$ to be measured. The accuracy of the $T_1$ values is better than ±15%. $T_1$ values were determined by a least-squares fit to the best straight line. The sample under study was placed in a tube of 12 mm o.d; chemical shifts are reported downfield from external tetramethylsilane contained in a concentric inner tube of 5 mm o.d. For the experiments in $H_2O$, the sample was placed in a tube of 10 mm o.d. The field-frequency lock was obtained by placing $D_2O$ in the concentric 12-mm outer tube. Spectra were taken at 32°.

## Results

*Determination of the Tautomeric Form of the Imidazole Ring of the* L-*Histidine Residue in TRF in Basic Solution.* The $^{13}$C chemical shifts of the histidine residue in TRF as a function of pH in $H_2O$ are shown in Figure 2. The pH profile for this histidine residue is similar to that of free L-histidine (Reynolds *et al.*, 1973). It has been shown that in L-histidine the $N^{\tau}$-H tautomer (also called $N^{\epsilon}$ or $N^3$) is the predominant tautomeric form of the imidazole ring of histidine in basic solution (Reynolds *et al.*, 1973). Thus we conclude that in TRF the $N^{\tau}$-H tautomer is also predominant at high pH. The p$K_a$ value, determined from the average value of the individual p$K_a$'s, is 6.2 ± 0.1. This is in agreement with the value of 6.25 obtained for histidine in TRF by Grant *et al.* (1972) using potentiometric titrations. The p$K_a$ of histidine in TRF is the same as that of L-histidine monomer, 6.2 (Reynolds *et al.*, 1973), thereby implying no difference in the intramolecular interactions occurring in the His residue of TRF and L-histidine monomer.

Curves of $^{13}$C chemical shifts *vs.* "pH" were also obtained for TRF dissolved in $D_2O$. The p$K_a$ value of TRF obtained from these curves was 6.1 ± 0.1. The p$K_a$ values obtained from studies in $D_2O$ and $H_2O$ are similar because the difference between activities of hydrogen and deuterium ions at the glass electrode is approximately equal and opposite to the difference in activities of these ions with respect to the titratable group (Sachs *et al.*, 1971). Further studies were carried out in $D_2O$ solution.

*pH Effects on* $^{13}$*Chemical Shifts in TRF.* Figure 3 shows the effect of "pH" changes on the $^{13}$C chemical shifts of all the carbons in TRF. The major changes occur in the His residue as a result of the charge change on the imidazole ring brought about by lowering the "pH" from 9 to 4. However, small, long-

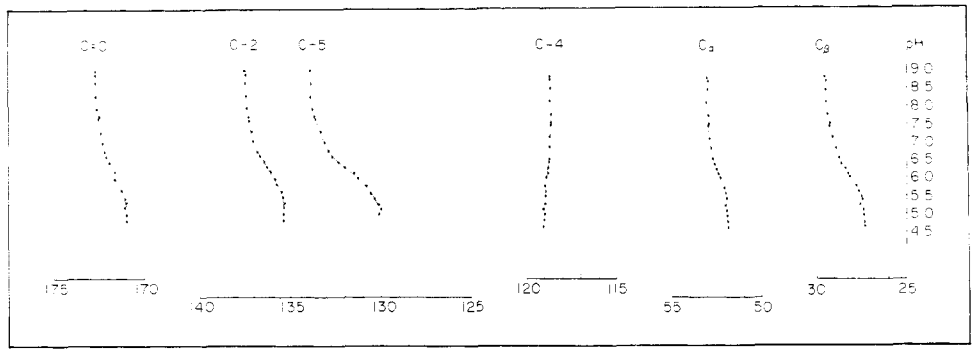


FIGURE 2: $^{13}$C chemical shifts of histidine in TRF as a function of pH. Chemical shifts are reported in parts per million downfield from external $Me_4Si$. Concentration of hormone, 250 mg/ml of $H_2O$.

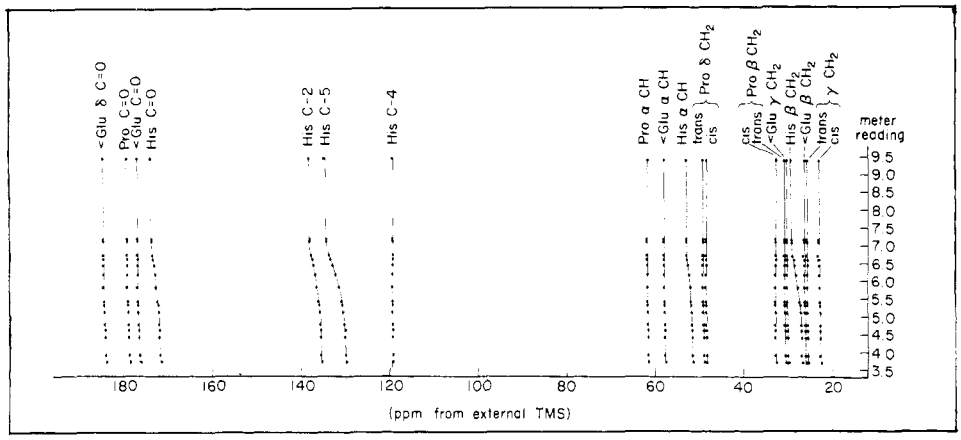


FIGURE 3: $^{13}$C chemical shifts of residues in TRF as a function of pH meter readings (pD = pH meter reading + 0.4) in $D_2O$. Concentration of hormone, 250 mg/ml. No effect of concentration on chemical shifts has been observed over the range 50–250 mg/ml.

range effects are noted on the <Glu and Pro residues. The $\beta$ carbon of <Glu shifts downfield 0.20 ppm (Table I). Both the cis and trans isomers about the peptide bond of Pro in TRF are affected, the cis isomer being perturbed to a greater degree. $^1$H nmr spectra of the trans isomer of Pro in TRF also show changes in the $\delta$ proton chemical shifts as a result of protonating the imidazole ring of His (Blagdon *et al.*, 1973). These observations may result from slight changes in steric constraints imposed on the TRF as a result of the charge change on the imidazole ring.

*$^{13}$C Spin–Lattice Relaxation Time Measurements.* $^{13}$C spin-lattice relaxation times ($T_1$) for TRF have been determined at pH values of 9.9 and 4.2. $NT_1$ values for TRF, where $N$ is the number of directly bonded hydrogen atoms, are given in Table II. These provide information concerning the intramolecular mobility of the various residues in the peptide (Allerhand and Komoroski, 1973; Doddrell *et al.*, 1972; Allerhand and Oldfield, 1973; Saitô and Smith, 1973; Allerhand *et al.*, 1971a,b; Deslauriers *et al.*, 1973a,b, 1974a; Keim *et al.*, 1973a,b; Saitô and Smith, 1974). In the limit of extreme narrowing (rapid motion on the $^{13}$C nmr time scale) the greater the $NT_1$ value, the more mobile is the $^{13}$C moiety.

There is little effect of "pH" change on the relative mobilities of the individual residues in TRF as manifest in the $T_1$ values. Both at pH 9.9 and 4.2 the $\alpha$ carbons of His and Pro appear to be equally mobile whereas the $\alpha$ carbon of the <Glu residue is more mobile than either of the other two $\alpha$ carbons. We calculate an effective correlation time ($\tau_c$) (Allerhand *et al.*, 1971b; Allerhand and Komoroski, 1973) of $1.2 \times 10^{-10}$ sec for reorientation of the TRF molecule based on the value of 0.4 sec for the $\alpha$ carbon of the His residue.

The side chain of His shows a slight increase in $NT_1$ values going from the $\alpha$ carbon to the protonated carbons of the imidazole ring. This demonstrates that the side chain of His under-

TABLE I: $^{13}$C Chemical Shifts[a] of TRF and Methylated Derivatives of TRF in $D_2O$ and $CD_3OH$.

| | | $\tau$-Me-His TRF | | | TRF | | | $\pi$-Me-His TRF | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $D_2O$ 4.7 | $D_2O$ 8.5 | $CD_3OH$ | $D_2O$ 4.7 | $D_2O$ 8.9 | $CD_3OH$ | $D_2O$ 4.7 | $D_2O$ 8.6 | $CD_3OH$ |
| <Glu | $\alpha$CH | 57.80 | 57.70 | 57.90 | 57.75 | 57.70 | 58.00 | 57.85 | 57.80 | 58.00 |
| | $\beta CH_2$ | 26.35 | 26.25 | 26.50 | 26.45 | 26.25 | 26.50 | 26.45 | 26.25 | 26.50 |
| | $\gamma CH_2$ | 30.30 | 30.15 | 30.40 | 30.25 | 30.20 | 30.40 | 30.40 | 30.25 | 30.30 |
| | $\delta$C=O | 183.30 | 183.35 | 181.85 | 183.25 | 183.10 | 181.90 | 183.35 | 183.35 | 181.90 |
| | C=O | 175.70 | 175.60 | 174.70 | 175.80 | 175.45 | 174.70 | 175.80 | 175.65 | 175.00 |
| His | $\alpha$CH | 51.65 | 52.90 | 53.20 | 51.85 | 52.90 | 53.30 | 50.70 | 51.45 | 51.25 |
| | $\beta CH_2$ | 27.20 | 30.00 | 30.40 | 27.10 | 29.30 | 29.90 | 26.45 | 26.05 | 26.60 |
| | C-2 | 136.40 | 139.60 | 139.05 | 134.85 | 137.20 | 137.10 | 137.00 | 140.40 | 139.60 |
| | C-4 | 122.80 | 120.60 | 120.40 | 118.90 | 118.35 | 119.10 | 119.25 | 128.00 | 128.10 |
| | C-5 | 130.10 | 136.40 | 137.50 | 129.50 | 133.90 | 134.10 | 131.25 | 128.70 | 128.90 |
| | C=O | 171.00 | 172.70 | 172.50 | 170.85 | 172.50 | 172.50 | 170.50 | 171.65 | 171.40 |
| | $CH_3$ | 36.75 | 34.15 | 33.80 | | | | 34.40 | 32.30 | 31.90 |
| Pro | $\alpha$CH | 61.55 | 61.60 | 61.75 | 61.55 | 61.50<br>61.40 | 61.70 | 61.75 | 61.70 | 61.65 |
| | $\beta CH_2$ cis | 33.05 | 32.50 | 33.50 | 33.10 | 32.55 | | | 32.95 | |
| | trans | 30.75 | 30.55 | 30.65 | 30.75 | 30.55 | 30.70 | 30.70 | 30.75 | 30.85 |
| | $\gamma CH_2$ trans | 25.95 | 25.60 | 25.70 | 25.75 | 25.65 | 25.70 | 25.80 | 25.75 | 25.85 |
| | cis | 22.90 | 23.10 | | 22.85 | 22.90 | | 23.00 | 23.05 | |
| | $\delta CH_2$ trans | 49.15 | 49.10 | 48.70 | 49.15 | 48.90 | 48.70 | 49.15 | 49.10 | 48.80 |
| | cis | 48.65 | 48.25 | | 48.60 | 48.20 | | 48.60 | 48.65 | |
| | C=O | 177.80 | 177.95 | 177.35 | 177.80 | 177.75 | 177.40 | 177.85 | 177.85 | 177.10 |

[a] Chemical shifts are reported in parts per million downfield from external $Me_4Si$. Accuracy is 0.05 ppm.

TABLE II: $NT_1$ Values[a] of the Carbon Resonances in TRF.[b]

| | | $D_2O$ | | |
|---|---|---|---|---|
| | | pH 9.9 | pH 4.7 | $(CD_3)_2SO$ |
| <Glu | $\alpha CH$ | 0.58 | 0.51 | 0.28 |
| | $\beta CH_2$ | 0.89 | 0.77 | 0.39 |
| | $\gamma CH_2$ | 0.82 | 0.83 | 0.41 |
| | $\delta C{=}O$[c] | 16.0 | 12.9 | |
| | $C{=}O$[c] | 8.8 | 8.5 | |
| His | $\alpha CH$ | 0.39 | 0.35 | 0.20 |
| | $\beta CH_2$ | 0.42 | 0.41 | 0.31 |
| | C-2 | 0.51 | 0.50 | |
| | C-4 | 0.59 | 0.46 | |
| | C-5[c] | 8.4 | 4.2 | |
| | $C{=}O$[c] | 7.4 | 8.3 | |
| Pro | $\alpha CH$ | 0.42 | 0.38 | 0.18 |
| | $\beta CH_2$ | 0.73 | 0.81 | 0.36 |
| | $\gamma CH_2$ | 0.86 | 0.79 | 0.41 |
| | $\delta CH_2$ | 0.52 | 0.46 | 0.26 |
| | $C{=}O$[c] | 11.8 | 8.9 | |

[a] $NT_1$ values are reported in seconds. [b] Concentration of TRF, 200 mg/ml. [c] $T_1$ values.

goes a slightly greater degree of motion than does the $\alpha$ carbon of His situated in the peptide backbone.

$T_1$ measurements of TRF in $(CD_3)_2SO$ (Table II), which can only accept hydrogen bonds, showed a decrease of a factor of 2 in all the $T_1$ values measured, but the relative ratios of $T_1$ values for the various carbons in the molecule were the same as those observed in $D_2O$. The decrease in $T_1$ values reflects a decrease in the overall rate of molecular reorientation which could be due to a change in microscopic viscosity or formation of a unit composed of two molecules. Changes in macroscopic viscosity cannot be responsible for the decrease in $T_1$ values because the viscosities of dimethyl sulfoxide and $D_2O$ are comparable (Stecher, 1968; Prutton and Maron, 1951). However, in either case, the lack of change in the *relative* $T_1$ values implies that the relative rates of intramolecular motion are the same in $(CD_3)_2SO$ as in $D_2O$.

*Methylated Derivatives of TRF.* The $^{13}C$ chemical shifts of $N^{\tau}$-methylimidazole TRF and $N^{\pi}$-methylimidazole TRF in $D_2O$ at pH 8.7 and 4.7 are given in Table I. At pH 8.7 the highly biologically active $N^{\tau}$-methylimidazole TRF (Vale *et al.*, 1971) differs from TRF mainly in the imidazole ring carbon resonances which are shifted downfield by $\simeq$2.5 ppm. The almost inactive $N^{\pi}$-methylimidazole TRF (Vale *et al.*, 1971) shows differences of up to 9.6 ppm in the imidazole ring carbon resonances when compared with TRF. At pH 4.7, where the methylimidazole rings of the TRF derivatives are protonated, the chemical shifts of the methylhistidine residues are more similar to those of the His residue in TRF at the same pH. This is particularly true for $N^{\pi}$-methylimidazole TRF. The $pK_a$ values determined by Grant *et al.* (1972) for $N^{\tau}$-methylimidazole TRF (5.95) and $N^{\pi}$-methylimidazole TRF (6.6) are in qualitative agreement with those determined by Reynolds *et al.* (1973) for free $N^{\tau}$-methylhistidine (6.3) and for free $N^{\pi}$-methylhistidine (6.7). Any postulate of intramolecular interaction involving the His residue in TRF or in $N^{\pi}$-methylimidazole TRF based on $pK_a$ values (Grant *et al.*, 1972) must also lead to similar postulates for free histidine and $N^{\tau}$-methylhistidine monomer. However, one cannot neglect the possibility that different electron densities on the nitrogen atoms of histidine, $N^{\pi}$-methylhistidine, and $N^{\tau}$-methylhistidine are correlated with the degree of affinity of the imidazole ring for a proton (and thus with the $pK_a$), even in the absence of intramolecular hydrogen bonds.

The changes in $^{13}C$ chemical shifts which occur upon imidazole ring protonation of His in TRF, $N^{\pi}$- and $N^{\tau}$-methylimidazole TRF are given in Table III, these values are comparable to those found for free His and free $N^{\pi}$- *and* $N^{\tau}$-methylhistidine; there is apparently little contribution from the adjacent <Glu and Pro-$NH_2$ residues.

*Spectra of TRF and N-Methylimidazole TRF in a Nonaqueous Solvent.* The chemical shifts of TRF, $N^{\pi}$- and $N^{\tau}$-methylimidazole TRF in $CD_3OH$ are given in Table I. The chemical shifts are almost identical with those observed in $D_2O$ implying no significant differences in conformation. Only the $\delta$ carbonyl resonance of <Glu shifts $\simeq$1.0 ppm which could be a consequence of greater solvent accessibility for this carbonyl when compared with the other carbonyl residues in TRF.

## Discussion

Minimum energy calculations have yielded a number of possible conformations for the TRF molecule (George and Kier, 1973; Blagdon *et al.*, 1973; Burgess *et al.*, 1973; Belle *et al.*, 1972). These models share some common features: the pyrrolidone ring is not involved in any intramolecular interaction, the His-Pro peptide bond is in the trans conformation, and there is no interaction between the cyclic portions of the peptide. Several intramolecular hydrogen bonds have been proposed. Blagdon *et al.* (1973) proposed a "hairpin turn" with a hydrogen bond between $N^{\pi}$ of the imidazole ring and the peptide NH of His; another hydrogen bond was included between a carboxamide proton of Pro and the peptide carbonyl oxygen of < Glu. Belle *et al.* (1972) found conformations in which hydrogen bonding could occur between the carbonyl oxygen of His and carboxamide proton of Pro; they also indicated attractive forces between the carbonyl oxygen of His and the imidazole $N^{\pi}$-H. The results of Burgess *et al.* (1973) suggested an extended conformation for TRF with a hydrogen bond between the carbonyl oxygen of His and a carboxamide proton of Pro. George and Kier (1973) found that the conformational preferences of individual amino acids calculated separately were reflected in the conformation of the whole molecule, only the angle about the $N_i$-$C_i^{\alpha}$-$C_i$-$N_{i+1}$ bonds ($\psi$) of His changed by 180 °.

Grant *et al.* (1972) using potentiometric titration data, suggested hydrogen bonding between $N^{\pi}$ of the imidazole ring and the peptide N-H of His. Fermandjian *et al* (1972), using $^1H$ nmr chemical shift data, proposed a hydrogen bond between the trans carboxamide proton of Pro and the carbonyl oxygen of His. However, no temperature dependence of N-H proton chemical shifts was determined (hydrogen-bonded amide proton resonances exhibit smaller upfield shifts as a function of increasing temperature than those observed for amide protons which are exposed to solvent (Urry and Ohnishi, 1970)). Furthermore the amide protons in acetylprolinamide in $(CD_3)_2SO$ do not exhibit the large chemical shift nonequivalence, nor the weak temperature dependence (R. Deslauriers, unpublished) characteristic of hydrogen bonding in peptides. $^1H$ nmr spectra cannot provide information regarding the hydrogen-bond acceptor; therefore the carbonyl oxygen of the <Glu residue

[3] The [$N^{\tau}$-Me-His][2] derivative of luteinizing hormone-releasing hormone (LH-RH) does not show biological activity superior to that of LH-RH (6% activity of LH-RH) and [$N^{\pi}$-Me-His][2] LH-RH has 2% of the activity of LH-RH. This indicates that such changes in the structure of His in another hypothalamic factor do not have the same beneficial effects (Monahan *et al.*, 1972).

TABLE III: Chemical Shift Changes[a] Occurring upon Protonation of His and Methylated Histidine in Amino Acid[b] and TRF.

| Histidine Derivative | Position | | | | | |
|---|---|---|---|---|---|---|
| | $C_2$ | | $C_4$ | | $C_5$ | |
| | Amino Acid | TRF | Amino Acid | TRF | Amino Acid | TRF |
| $\tau$-Me-His | −3.5 | −3.2 | +2.1 | +2.2 | −6.8 | −6.3 |
| His | −2.4 | −2.4 | +0.7 | +0.6 | −4.8 | −4.4 |
| $\pi$-Me-His | −3.4 | −3.4 | −7.1 | −8.7 | +2.3 | +2.5 |

[a] Chemical shift changes are reported in parts per million. Negative sign indicates upfield shift. [b] Reynolds *et al.*, 1973.

could as easily be involved in hydrogen bonding to the amide proton of Pro (Deslauriers *et al.*, 1973b).

In our previous $^{13}$C nmr studies on TRF we detected *both* cis and trans isomers about the His-Pro peptide bond (Deslauriers *et al.*, 1973a; Smith *et al.*, 1973) in TRF. The relative proportions of cis and trans isomers varied with the solvent and the proportion of the trans isomer was greater than that observed in Pro-containing dipeptides (Thomas and Williams, 1972) or in acetylprolinamide (Deslauriers *et al.*, 1973a). We suggested that the increase in proportion of the trans His-Pro isomer in TRF, relative to the dipeptides, is the consequence of steric constraints introduced by the <Glu-His moiety (Deslauriers *et al.*, 1973b). The observations and the $T_1$ data (*vide supra*) demonstrate that a number of conformations are possible for TRF; the theoretical calculations of a number of minimum energy conformations mentioned previously support this statement further.

The present $T_1$ measurements have shown that the His-Pro moiety is more rigid than the <Glu residue. Rotation about the $C_\alpha$-CO bond of <Glu is possible. Similar conclusions have been drawn by Burgess *et al.* (1973) from minimum energy calculations. The side chain of His is slightly more mobile than the peptide backbone in the His-Pro moiety. The proposed hydrogen bond between the $N^\pi$ of the imidazole ring and the peptide N-H of His (Grant *et al.*, 1972), if it exists, does not restrict the motion of the His side chain, since breaking this bond by protonating the imidazole ring does not result in any changes in $T_1$ values for the His side chain. It must therefore be at most a minor conformer of TRF. The $T_1$ values for the $\beta$, $\gamma$, and $\delta$ carbons of Pro suggest intracyclic motion. The ring conformation is best described as a rapidly interconverting set of half-chair conformers where the $\beta$ and $\gamma$ carbons undergo the greatest changes in position (Deslauriers *et al.*, 1974b).

In these studies we have shown that the $N^\tau$-H tautomer is the predominant form of the imidazole ring of His in TRH in basic solution. Theoretical calculations suggest that placing the hydrogen atom on either $N^\pi$ or $N^\tau$ does not cause any conformational changes (Burgess *et al.*, 1973); however, the position of the proton does seem to affect biological activity. We propose that the active form of TRF at the receptor is that in which the proton is situated on $N^\tau$ of the imidazole ring of His. At physiological pH approximately 90% of the His residues will be in the neutral form. In the neutral form of His the $N^\tau$-H:$N^\pi$-H tautomer ratio is approximately 4:1 (Reynolds *et al.*, 1973). Any type of change which alters the tautomer ratio in favor of $N^\tau$-H or decreases the $pK_a$ of His should increase the intrinsic activity of TRF, assuming that changes do not occur in the conformation required at the receptor. The biologically hyperactive $N^\tau$-methylimidazole TRF has been calculated to have the same conformation as TRF (Burgess *et al.*, 1973), a $pK_a$ of 5.95 (Grant *et al*, 1972), but in the former the fixed $N^\tau$-$CH_3$ bond eliminates tautomerism and keeps the His in the active form. Hydrophobic interactions at the receptor may also be important, in view of the activity of $N^\tau$-methylimidazole TRF;[2] such interactions have been proposed for the neurohypophyseal hormones, oxytocin and vasopressin. Neurohypophyseal hormones are thought to interact with their receptors in a manner which allows the "hydrophobic surface of the molecule to be in close contact with the hydrophobic cleft of the receptor, while the opposite surface, containing the hydrophilic groups of the hormone faces away from the cleft of the receptor toward the aqueous environment" (Walter *et al.*, 1971).

Synthesis and biological assay of derivatives which have the above mentioned properties should provide a good test of this proposed hypothesis.

## References


Allerhand, A., Doddrell, D., Glushko, V., Cochran, D. W., Wenkert, E., Lawson, P. J., and Gurd, F. R. N. (1971a), *J. Amer. Chem. Soc. 93,* 544-546.

Allerhand, A., Doddrell, D., and Komoroski, R. (1971b), *J. Chem. Phys. 55,* 189-198.

Allerhand, A., and Komoroski, R. A. (1973), *J. Amer. Chem. Soc. 95,* 8228-8231.

Allerhand, A., and Oldfield, E. (1973), *Biochemistry 12,* 3428-3433.

Belle, J., Montagut, M., and Bellocq, A. M. (1972), *C. R. Acad. Sci., Ser. C 275,* 471-474.

Bellocq, A. M., Boilot, J. C., Dupart, E., and Dubien, M. (1973), *C. R. Acad. Sci., Ser. D 276,* 423-426.

Blagdon, D. E., Rivier, J., and Goodman, M. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1166-1168.

Boilot, J. C. (1972), Thèse Docteur-Ingénieur Université de Bordeaux I.

Bowers, C. Y., Weil, A., Chang, J. K., Sievertsson, H., Enzmann, F., and Folkers, K. (1970), *Biochem. Biophys. Res. Commun. 40,* 683-691.

Burgess, A. W., Momany, F. A., and Scheraga, H. A. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1456-1460.

Burgus, R., Dunn, T. F., Desiderio, D. M., and Guillemin, R. (1969a), *C. R. Acad. Sci., Ser. D 269,* 1870-1873.

Burgus, R., Dunn, T. F., Ward, D. N., Vale, W., Amoso, M., and Guillemin, R. (1969b), *C. R. Acad. Sci., Ser. D 268,* 2116-2118.

Chang, J. K., Sievertsson, H., Currie, B., Folkers, K., and Bowers, C. Y. (1971), *J. Med. Chem. 14,* 484-487.

Deslauriers, R., Garrigou-Lagrange, C., Bellocq, A. M., and Smith, I. C. P. (1973a), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 31,* 59-66.

Deslauriers, R., Smith, I. C. P., and Walter, R. (1974a), *J. Amer. Chem. Soc. 96,* 2289.

Deslauriers, R., Walter, R., and Smith, I. C. P. (1973b), *Biochem. Biophys. Res. Commun. 53,* 244-250.

Deslauriers, R., Walter, R., and Smith, I. C. P. (1974), *J. Biol. Chem.* (in press).
Doddrell, D., Glushko, V., and Allerhand, A. (1972), *J. Chem. Phys. 56,* 3683–3689.
Enzmann, F., Bøler, J., Folkers, K., Bowers, C. Y., and Schally, A. V. (1971), *J. Med. Chem. 14,* 469–474.
Fermandjian, S., Pradelles, P., and Fromageot, P. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28,* 156–160.
Folkers, K., Enzmann, F., Bøler, J., Bowers, C. Y., and Schally, A. V. (1969), *Biochem. Biophys. Res. Commun. 37,* 123–126.
Freeman, R., and Hill, H. D. W. (1970), *J. Chem. Phys. 54,* 3367–3377.
George, J. M., and Kier, L. B. (1973), *J. Theor. Biol. 40,* 393–396.
Glascoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64,* 188–190.
Grant, G., Ling, N., Rivier, J., and Vale, W. (1972), *Biochemistry 11,* 3070–3073.
Keim, P., Vigna, R. A., Marshall, R. C., and Gurd, F. R. N. (1973a), *J. Biol. Chem. 248,* 6104–6113.
Keim, P., Vigna, R. A., Morrow, J. S., Marshall, R. C., and Gurd, F. R. N. (1973b), *J. Biol. Chem. 248,* 7811–7818.
Monahan, M., Rivier, J., Vale, W., Ling, N., Grant, G., Amoss, M., Guillemin, R., Burgus, R., Nicolaides, E., and Rebstock, M. (1972), *in* Chemistry and Biology of Peptides, Meienhofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, pp 601–608.
Prutton, C. F., and Maron, S. H. (1951), Fundamental Principles of Physical Chemistry, New York, N. Y., Macmillan, p 679.
Reynolds, W. F., Peat, I. R., Freedman, M. H., and Lyerla, J. R., Jr. (1973), *J. Amer. Chem. Soc. 95,* 328–331.
Rivier, J., Vale, W., Monahan, M., Ling, N., and Burgus, R. (1972), *J. Med. Chem. 15,* 479–482.
Sachs, D. H., Schechter, A. N., and Cohen, J. S. (1971), *J. Biol. Chem. 246,* 6576–6580.
Saitô, H., and Smith, I. C. P. (1973), *Arch. Biochem. Biophys. 158,* 154–163.
Saitô, H., and Smith, I. C. P. (1974), *Arch. Biochem. Biophys.* (in press).
Schally, A. V., Arimura, A., Bowers, C. Y., Kastin, A. J., Sawano, S., and Redding, T. S. (1968), *Recent Progr. Hormone Res. 24,* 497–588.
Smith, I. C. P., Deslauriers, R., Saitô, H., Walter, R., Garrigou-Lagrange, C., McGregor, H., and Sarantakis, D. (1973), *Ann. N. Y. Acad Sci. 222,* 597–627.
Stecher, P. G., Ed. (1968), The Merck Index, Rahway, N. J., Merck and Co., p 378.
Thomas, W. A., and Williams, M. K. (1972), *J. Chem. Soc., Chem Commun.,* 994.
Urry, D. W., and Ohnishi, M. (1970), *in* Spectroscopic Approaches to Biomolecular Conformation, Urry, D. W., Ed., Chicago, Ill., American Medical Association, pp 263–300.
Vale, W., Rivier, J., and Burgus, R. (1971), *Endocrinology 89,* 1485–1488.
Walter, R., Schwartz, I. L., Darnell, J. H., and Urry, D. W. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1355–1359.

# Rhodopsin. Purification and Recombination with Phospholipids Assayed by the Metarhodopsin I → Metarhodopsin II Transition†

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ABSTRACT: Studies of the nature of interaction between the visual protein rhodopsin and the rod outer segment (ROS) membrane phospholipid components have been initiated. To assay this interaction, a flash photolysis instrument has been built with microsecond resolution allowing the kinetic observation of the spectroscopic intermediates metarhodopsin $I_{480\,nm}$ → metarhodopsin $II_{380\,nm}$ in the bleaching process of rhodopsin. A single first-order rate has been established for the kinetic appearance of metarhodopsin $II_{380\,nm}$ in preparations of rhodopsin in its native disc membrane environment (ROS membranes) and for dodecyldimethylamine oxide (DDAO) detergent solubilized rhodopsin. A purification procedure has been developed for the preparation of rhodopsin free of phospholipid and detergent and the isolated protein can be recombined with phospholipids to obtain a "reconstituted" lipid–protein species of defined composition. The spectroscopic assay is a sensitive indication of the protein–lipid interaction. The following lifetimes for metarhodopsin I → metarhodopsin II were observed at 20°: sonicated ROS membranes, 20 msec; purified lipid-free rhodopsin in DDAO, 0.08 msec; rhodopsin reassembled with egg phosphatidylcholine, 9 msec (70% component), 2 msec (30% component). The transition is blocked for rhodopsin free of detergent and lipid but can be restored by addition of detergent.

The molecular photoreceptor in both vertebrate and invertebrate rod cells of the retina has been shown to be the chromoprotein rhodopsin. This membrane protein contains the tightly coupled chromophore retinal which isomerizes from the 11-cis to the all-trans form upon absorption of light (Wald, 1968). The ultimate consequence of this absorption is the triggering of a bioelectrical activity in the cell which can be transmitted to higher order neurons (Sillman *et al.*, 1969; Hagins *et al.*, 1970, Hagins, 1972).

Following absorption of a photon a series of intermediate steps have been spectrally defined for this chromoprotein. The

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