THE INFLUENCE OF ORGANIC SOLVENTS ON THE CONFORMATION OF THYREOTROPIN RELEASING FACTOR (TRF) STUDIED BY CIRCULAR DICHROISM

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Thyreotropin releasing factor (TRF), L-pyroglutamyl-L-histidyl-L-prolinamide, and some of its analogues have been studied by circular dichroism in organic solvents. In dioxane-rich solutions, intramolecular hydrogen bonding occurs between an amidic proton of proline and the carbonyl group of the histidine residue, inducing a strong negative Cotton effect at 226 nm, an effect similar to that described for N-acetylprolinamide and other N-acetylaminoacid amides. The CD spectra of TRF and analogues in hexafluoro-2-propanol are compared with those of N-methylimidazole derivatives. The observations suggest that a linkage between the N^{π} side of the imidazole ring and another group in the molecule plays a role in the stability of the prolinamide conformation. It would appear that the tautomeric form of the imidazole ring is N^{τ}—H in both solvents.

In the past few years the synthetic peptide L-pyroglutamyl-L-histidyl-L-prolinamide, TRF, which exhibits all the biological activities of thyreotropin releasing factor[1,2] has been studied by proton and ^{13}C-NMR (ref.[3-7]), infra-red Raman spectroscopy[8] and semi-empirical conformational calculations[9-12]. In spite of these efforts, the TRF conformation is still a matter of controversy. This situation is not too surprising since TRF is made of three amino acids which, despite the bulkiness of their side chains, cannot be expected to offer much cooperativity in their possible mutual interactions. The role of the surrounding medium is therefore of paramout importance.

The present work reports a study by circular dichroism of TRF and some of its analogues, dissolved mainly in dioxane. This approach seemed appropriate after the fundamental data afforded by the investigations of Tsuboï and coworkers[13] and Madison and Schellman[14] on N-acetyl-L-prolinamide. These authors and Cann[15] have shown that N-acetyl-L-prolinamide, as well as other N-acetyl-L-amino acid amides, folded on themselves when the solvent became less polar. These model peptides, in dioxane-rich solvents, showed a strong negative ellipticity band peaking

around 230 nm. This band was attributed to the formation of a hydrogen bond between the acetyl oxygen and one hydrogen of the C-terminal amide.

In this paper we compare the CD spectra of TRF, its diastereoisomers and its methylamide derivatives with those of N-acetyl-L-prolinamides, in order to show that TRF mimics the folding of simple prolinamides. Further, the difference in the CD spectra of TRF, [N^{π}-Me-His^2]-TRF* and [N^{τ}-Me-His^2]-TRF is used to elucidate the role of the histidine side-chain in the spatial arrangement of the TRF molecule.

EXPERIMENTAL

Methods

Melting points were determined on a Kofler block. Samples for analysis were dried for 24 h at a pressure of 0·5 Torr over phosphorus pentoxide. Optical rotation was measured on photo-electric polarimeter Perkin Elmer 141. Purity of the substances was checked by thin-layer chromatography on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the solvent systems 2-butanol–formic acid–water (75 : 12 : 13); 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) and by electrophoresis on Whatman No 4 paper at pH 2·4; pH 5·7 at a potential gradient of about 20 V/cm.

The circular dichroic curves were recorded between 260—200 nm at room temperature using a Jouan Dichrograph II with a Xenon lamp. The range of concentration of peptides used was 10^{-3} to 10^{-4}M. Quartz cells (Hellma) had optical paths ranging from 0·01 to 1 cm. The molar ellipticities $[\Theta]$ are expressed in degree cm^2 dmol^{-1}.

Materials

The synthetic peptides: TRF, [Lys^2]-TRF, [3-Pyr-Ala^2]-TRF, [Pro^1]-TRF and L-Glu-L-His-OH were generously supplied by Dr R. O. Studer (Hoffman-La Roche, Basel). N-Acetyl-L-prolinamide and N′-monomethylamide, [N^{π}-Me-His^2]-TRF, [N^{τ}-Me-His^2]-TRF, TRF-N′-mono and N′,N′-dimethylamide were purchased from Bachem (U.S.A.). Dioxane and acetonitrile of spectroscopic grade from Merck were used without further purification, as was hexafluoro-2-propanol from Schuchardt.

Synthesis of [D-Glu^1]-TRF and of [D-Pro^3]-TRF

D-Pyroglutamic acid: A suspension of D-glutamic acid γ-methyl ester (336 mg) in methanol (30 ml) was saturated with ammonia until the ester dissolved. After standing for 24 h at room temperature the solution was evaporated, ethanol (30 ml) was added, the mixture was evaporated again and the residue dissolved in water (5 ml). The latter solution was filtered through a column of Dowex 50 (H^+ cycle, 16 ml). The ion-exchanger was washed with water (30 ml) and the pooled eluates were evaporated. The remainder was dried azeotropically with acetone and benzene and washed with ether to yield 204 mg (71%) of D-pyroglutamic acid, m.p .152—156°C; $[\alpha]_D^{25}$ +11·2° (*c* 1, water). Ref.[18] for L-isomer m.p. 159—160·5°C; $[\alpha]_D^{20}$ —11·7° (*c* 4, water).

* For Symbols and nomenclature of peptides see ref.[16,17]. TRF is used for the thyreotropin releasing factor, 3-Pyr-Ala for 2-amino-3-(1-pyrrolyl)propionic acid.

D-*Pyroglutamyl*-L-*histidinhydrazide*: To D-pyroglutamic acid (200 mg) in dimethylformamide (6 ml) L-histidine methyl ester freed from 333 mg of the dihydrochloride with ammonia in chloroform and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (428 mg) were added. After 7 h a further portion of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (95 mg) was added and the mixture was left at room temperature overnight. After evaporation the residue was triturated three times with ether, dissolved in methanol (2·5 ml), cooled to −15°C and 99% hydrazine (0·25 ml) was added. The solution was left overnight at 5°C. The separated crystals were collected and washed with ethanol and ether to yield 279 mg (72% calculated on the dihydrochloride of histidine methyl ester) of D-pyroglutamyl-L-histidinhydrazide, m.p. 238–241°C (dec.). Recrystallization from water–ethanol yielded 226 mg (58%) of the product, m.p. 245–248°C (dec.); $[\alpha]_D^{25}$ −7·96° (*c* 0·48, water). For $C_{11}H_{16}N_6O_3$ (280·3) calculated: 47·13% C, 5·75% H, 29·99% N; found: 47·19% C, 5·75% H, 30·14% N.

D-*Pyroglutamyl*-L-*histidyl*-L-*prolinamide*: The coupling step was carried out according to[19]. D-Pyroglutamyl-L-histidinhydrazide (143 mg) was suspended in dimethyl sulphoxide (2 ml) and dimethylformamide (3 ml). A solution of hydrogen chloride in tetrahydrofurane (2·78M, 1·08 ml) was added with stirring at 0°C. The mixture was cooled to −30°C and butyl nitrite (0·10 ml) was added over 1 min. After 3 min stirring at −30°C L-prolinamide (62 mg) and triethylamine (0·42 ml) were added. The mixture was stirred at −20°C for 30 min and left at 5°C overnight. Triethylamine hydrochloride was filtered off, dimethylformamide was evaporated off and the residue was triturated by adding ether–tetrahydrofurane 1 : 1 (40 ml). The precipitate was dissolved in methanol (2 ml) and precipitated again in the same way to yield 160 mg of a product which was purified on a column of CM-Sephadex (12·5 ml) in the buffer acetic acid–pyridine–water (2·5 : 10 : 1000), pH 5·7. Fractions containing D-pyroglutamyl-L-histidyl-L-prolinamide (localised preliminarily with Pauly reagent and then more exactly with Folin-Ciocalteau reagent) were pooled and evaporated. The residue was lyophilised from water yielding 63 mg (35%) of the acetate of D-pyroglutamyl-L-histidyl-L-prolinamide, $[\alpha]_D^{25}$ −62° (*c* 0·38, 1M acetic acid). For $C_{16}H_{22}N_6O_4.C_2H_4O_2.1/2\,H_2O$ (431·4) calculated: 50·11% C, 6·30% H, 19·47% N; found: 49·52% C, 6·25% H, 19·49% N. Amino-acid analysis: Glu 1·0, His 1·01, Pro 1·03.

D-*Prolinamide:* To benzyloxycarbonyl-D-proline (330 mg) in chloroform (4 ml) 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (400 mg) and 2·5M ammonia in chloroform (0·66 ml) were added at 0°C. After standing 1 h at 0°C the mixture was left overnight at room temperature. The solvent was evaporated off and the residue was triturated with ether to yield 240 mg (73%) of benzyloxycarbonyl-D-prolinamide, m.p. 90–91°C, $[\alpha]_D^{25}$ +33·5° (*c* 0·5, ethanol); ref.[20] m.p. 94°C, $[\alpha]_D^{23}$ +33·6° (*c* 2, ethanol). Benzyloxycarbonyl-D-prolinamide (240 mg) was treated with a 35% solution of hydrogen bromide in acetic acid (3 ml) at room temperature for 30 min, the solution was evaporated, the residue was triturated with ether and dissolved in 50% methanol (5 ml), and the solution was passed through a column of Dowex 50 (H^+ cycle, 3 ml). The ion exchanger was washed with 50% methanol and prolinamide was eluated with 3% ammonia in 50% methanol. The eluate was evaporated and the residue was dried azeotropically with benzene to yield 94 mg (62% calculated on benzyloxycarbonyl-D-proline) of D-prolinamide, m.p. 95–97°C, ref. for L-isomer 99°C (ref.[20]), 102–104°C (ref.[21]).

L-*Pyroglutamyl*-L-*histidyl*-D-*prolinamide*: The synthesis was carried out as described for D-pyroglutamyl-L-histidyl-L-prolinamide. Yield 34%. For $C_{16}H_{22}N_6O_4.C_2H_4O_2.1/2\,H_2O$ (431·4) calculated: 50·11% C, 6·30% H, 19·47% N; found: 50·27% C, 6·39% H, 19·66% N. Amino-acid analysis: Glu 1·0, His 1·05, Pro 0·93.

RESULTS AND DISCUSSION

Fig. 1 shows the CD spectrum of TRF in aqueous solution at pH 9. When dioxane is added, and its concentration increased above 70% (v : v), the spectrum changes drastically, and a negative band located between 225–230 nm appears, as shown in Fig. 2. This spectral change is fully reversible when the dioxane concentration is reduced. The behaviour of TRF in this respect is comparable to that of N-acetyl--L-prolinamide, which also presents a negative band in the same wavelength range when dissolved in solvents of low polarity as described by Tsuboï and coworkers[13] and by Madison and Schellman[14]. Cann[15] has presented several other examples of N-acetylamino acid amides, the CD spectra of which exhibit a negative band between 220–230 nm in dioxane solution, but not in water. These data suggest that the negative band at 226 nm, observed in the TRF spectrum, indicates an $n-\pi^*$ transition of the prolinamide carbonyl group involved in an intramolecular hydrogen bond. To examine the extent of the analogy between TRF and N-acetyl-L-prolinamide, a closer analysis was carried out by comparing the N′-mono- and N′,N′-dimethyl-

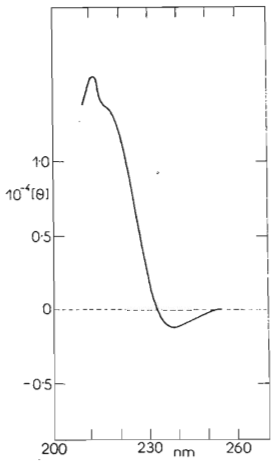

Fig. 1.
CD Spectrum of TRF in Aqueous Solution at pH 9, 0·1M Sodium Hydrogen Carbonate Buffer

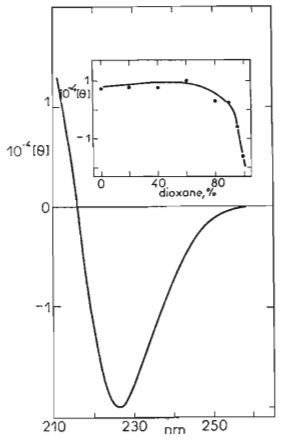

FIG. 2
CD Spectrum of TRF in Dioxane
The insert shows the variation of the negative band as a function of dioxane concentration.

amide derivatives of TRF and N-acetyl-L-prolinamide respectively. Fig. 3 shows the CD spectra of the two families of compounds dissolved in dioxane. The CD curves of TRF and N-acetyl-L-prolinamide and their methylamides are strikingly similar. Both the non-methylated and the monomethyl derivatives, which are able to hydrogen bond, show a negative Cotton effect between 220–230 nm. The blue shift of the band, described by Cann[15] for the monomethylamide of N-acetyl-L-proline, is also observed with TRF methylamide. On the other hand, when both amide protons are substituted the negative band disappears.

The close similarity between the CD spectra of both families of compounds suggests in addition that the imidazole and pyroglutamyl chromophores do not contribute much in the wavelength range considered. The 4 nm blue shift of the TRF negative band, compared to that of the reference compounds, might reflect either hindrance in solvation afforded by the bulky environment of the proline peptide linkage or a higher content of the folded conformation. These data indicate that the 226 nm negative band in TRF corresponds to a Cotton effect, presumably of the same type as that described for N-acetyl-L-prolinamide. It should be remembered, however, that in N-acetyl-L-prolinamide there is only one possibility for intramolecular binding of one hydrogen of the amide group, a positioning of the N-acetylproline peptide group in *trans* conformation and orientation of the amide proton towards the acetyl carbonyl. A seven-membered ring is closed in such a conformation. In TRF,

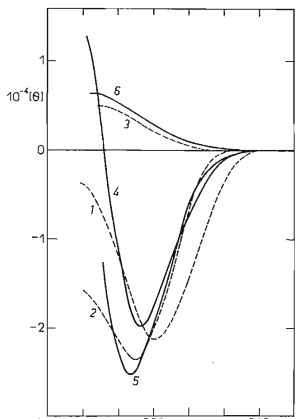

FIG. 3

CD Spectra of Methylated Amide Derivatives in Dioxane

1 N-Acetyl-L-prolinamide, *2* N-acetyl-L-prolin-N′-methylamide, *3* N-acetyl-L-prolin-N′,N′-dimethylamide (curve replotted from ref.[14]), *4* TRF, *5* TRF-N′-methylamide, *6* TRF-N′,N′-dimethylamide.

a comparable situation might occur but there is an other possibility: formation of a ten-membered ring involving the pyroglutamyl carbonyl. In order to choose between the possibilities which have been considered[3,9,11], CD spectra of several TRF analogues were analysed. In particular, replacing the L-pyroglutamyl residue by the D enantiomer should not appreciably alter the CD spectrum in the 220–230 nm range in the case of the seven-membered ring, since the pyroglutamyl residue is exterior to the chromophores under discussion. On the other hand, this substitution should drastically perturb the spectrum if the pyroglutamyl carbonyl group participated directly in fixation of the prolinamide terminus. Replacing the L-proline residue by the D enantiomer should in both cases invert the spectrum. Fig. 4 gives the results, indicating that the prolinamide residue plays a role whereas the pyroglutamyl residue is not involved in the folding which is reflected by the 226 nm negative band. This conclusion is supported by the removal of the carbonyl oxygen atom in the pyroglutamyl ring. The resulting compound, [Pro^1]-TRF, presents in dioxane a CD spectrum similar to that of TRF: ($[\Theta] \; -29 \,.\, 10^{-3}$ at 225 nm).

Thus it can be safely concluded that in dioxane, the C-terminus of TRF has a privileged conformation defined by a hydrogen bond between one amide proton and the carbonyl of histidine. This conformation requires that the His-Pro peptide grouping is *trans*. Infrared spectra of Z-His-Pro-NH_2 (in tetrachloromethane[6]) and of model compounds[22] support this conclusion. In acetonitrile the compounds under study

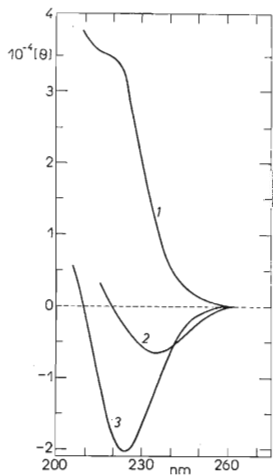

FIG. 4

CD Spectra of [D-Pro^3]-TRF *1*, [Lys^2]-TRF *2*, [D-Glu^1]-TRF *3* in 93, 98 and 98% (v : v) Dioxane–Water Solution, Respectively

give CD spectra (not shown) nearly identical to those in dioxane, reflecting the predominant conformation of the His-Pro-NH_2 portion in TRF. It should be kept in mind that the rotational energy barrier of the X-Pro peptide group is much lower than that of a peptide group not involving proline. Therefore the existence of other less frequent conformations (for instance *cis*) of the His-Pro-NH_2 moiety has been envisaged.

The question may be put as to whether the histidine side-chain plays a role in the stability of the prolinamide conformation. For this purpose [Lys^2]-TRF was investigated. Its CD spectrum in 98% dioxane (Fig. 4) presents a negative band at 235 nm about 2·5 times less intense than that exhibited by TRF at 226 nm. In the same solvent, N-acetyl-L-prolinamide has a negative peak at 230 nm. These features suggest that the [Lys^2]-TRF negative band is similar to that of TRF, and also reflects a hydrogen bonded conformation of the Pro-NH_2 moiety. However, since neither the imidazole chromophore nor the lysine side-chain give rise to a significant CD contribution in this region, the reduction in intensity of the negative band of [Lys^2]-TRF indicates that the population of the seven-membered ring is reduced. When the Pro-NH_2 residue is not stabilized by a seven-membered ring, as in the case of Pro-$N(CH_3)_2$ derivatives (Fig. 3), the ellipticity of the peptide is positive and increases from 250 towards 220 nm. Thus the shift of the negative band of [Lys^2]-TRF to 235 nm also reflects a reduction of the seven-membered ring contribution.

This concept is reinforced by considering the spectra of TRF and its analogues in hexafluoro-2-propanol. Hexafluoro-2-propanol is able to protonate[23] the imidazole ring and compete with the amide hydrogen in hydrogen bond conformation. In the CD spectra of L-[D]Glu-L-His-OH and TRF dimethylamide (recorded in hexafluoro--2-propanol, Fig. 5) a negative ellipticity peaking at 222 – 220 nm is present. However, TRF, [3-Pyr-Ala^2]-TRF, and [Pro^1]-TRF all exhibit more intense negative bands at 220 – 230 nm, indicating an additional contribution from the prolinamide moiety locked into the seven-membered ring. Again, in this solvent, the pyroglutamyl ellipticities do not overlap with those of histidine or prolinamide. [Lys^2]-TRF (Fig. 5) shows no negative ellipticity at all above 215 nm in hexafluoro-2-propanol, suggesting that the population of the folded conformation is too weak to be noticeable. Fig. 6 indicates that N-acetyl-L-prolinamide itself is only partially folded in hexafluoro-2-propanol. Comparison of Figs 5 and 6 leads to the conclusion that the nature of the amino acid preceding the prolinamide residue plays a role in the conformation of the latter, and that the imidazole side-chain exerts a stabilizing effect on the folding of the prolinamide moiety. To approach the problem of which part of the imidazole ring is responsible for this effect, N-methylimidazole derivatives were examined. The CD spectrum of [N^{τ}-Me-His^2]-TRF in dioxane (Fig. 7) shows a negative band at 226 nm similar in shape to that of TRF, but somewhat more intense. This shows that the N^{τ} atom can accomodate a bulky group without perturbating

the prolinamide location. The spectra of TRF and [N^{τ}-Me-His^2]-TRF given in Figs 2 and 7, are compatible with the suggestion[24] that the proton and the methyl group are both located on the same imidazole nitrogen atom, namely N^{τ}, since no differences in the spectra are noticed. The somewhat higher intensity of the negative band of [N^{τ}-Me-His^2]-TRF could reflect the inductive effect of the methyl group, which lowered the pK_a of the imidazole side-chain to 5·95, as compared to 6·25 for TRF (ref.[25]). By contrast, methylation of imidazole N^{π} atom leads to important spectral changes, as shown in Fig. 7. Since [N^{π}-Me-His^2]-TRF is less soluble than TRF in dioxane, 7% water (v : v) had to be added. Under these conditions, [N^{π}-Me-His^2]-TRF presents no negative band at 226 nm, but a positive ellipticity. A small negative band is observed at 240 nm.

Direct comparison of [N^{π}-Me-His^2]-TRF and [N^{τ}-Me-His^2]-TRF is not possible, since more than one variable was changed. More specifically, methylation of the

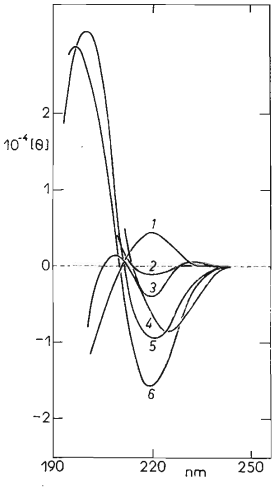

FIG. 5

CD Spectra of [Lys^2]-TRF *1*, L-Glu-L-His-OH *2*, TRF-N′,N′-dimethylamide *3*, [3-Pyr-Ala^2]-TRF *4*, TRF *5*, [Pro^1]-TRF *6* in Hexafluoro-2-propanol

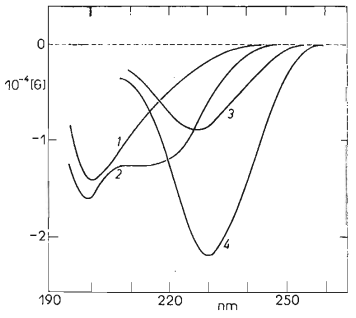

FIG. 6

CD Spectra of N-Acetyl-L-prolinamide in Water *1*, Hexafluoro-2-propanol *2*, Acetonitrile *3*, Dioxane *4*

imidazole N^{π} atom *a*) shifts the tautomer distribution, and *b*) might change the orientation of the side chain, and both these effects might result directly or indirectly in CD modifications. In order to simplify the situation, both methyl derivatives were examined in hexafluoro-2-propanol. This solvent will protonate the histidine side-chain and, presumably, „smooth out" the intrinsic differences between the two methylated forms. This point has been veryfied experimentally with free N^{π}-Me and N^{τ}-Me histidine, the CD curves of which (not shown) are virtually superimposeable in the protonated form and very different in the neutral form. Fig. 8 indicates that whereas the [N^{τ}-Me-His^2]-TRF CD spectrum resembles that of TRF as expected, the [N^{π}-Me-His^2]-TRF spectrum shows no negative band around 222 nm, rather a positive ellipticity. This result suggests that fixation of the prolinamide moiety in the seven-membered ring is no longer possible, at least to a detectable extent, when a methyl group is located on the imidazole N^{π} atom. This conclusion points again to the conformational role of the amino acid preceding the terminal prolinamide.

On this basis the CD curve of [N^{π}-Me-His^2]-TRF in dioxane can be interpreted as reflecting a large reduction in the population of the prolinamide folded conformation. Thus, each side of the histidine side-chain has a distinct position in space and

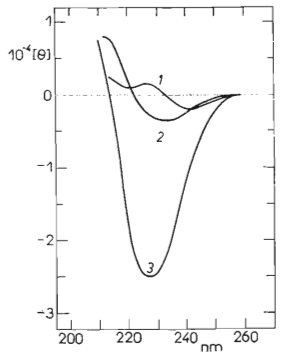

FIG. 7

CD Spectra of [N^{π}-Me-His^2]-TRF in 93% Dioxane *1*, [N^{τ}-Me-His^2]-TRF in 93% *2* and 100% *3* Dioxane, Respectively

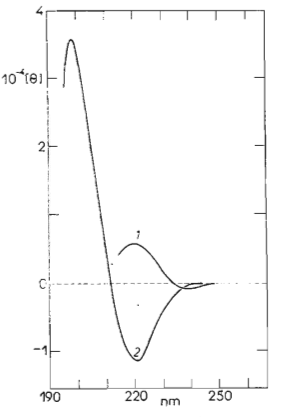

FIG. 8

CD Spectra of [N^{π}-Me-His^2]-TRF *1* and [N^{τ}-Me-His^2]-TRF *2* in Hexafluoro-2-propanol

no free rotation is allowed. The N^{τ} side appears to be surrounded by an „empty area", whereas the N^{π} side is close to an area critical for prolinamide folding.

Finally there is a negative band located at 238–240 nm in the spectrum of [N^{π}-Me-His^2]-TRF dissolved in dioxane, in hexafluoro-2-propanol (as shown in Figs 7, 8) and also in the spectrum of TRF dissolved in aqueous buffer, pH 9. This negative band located at high wavelengths is observed in the series of TRF analogues when *a*) prolinamide is present at the C terminal and *b*) no intense negative band is located around 226 nm, presumably because when the latter is present, the former is not directly distinguishable. Moreover, N-acetyl-L-prolin-N',N'-dimethylamide and poly-L-proline I, in *cis* conformation, present a similar negative band when dissolved in cyclohexane[24] and 1-propanol[25], respectively. These data suggest that the negative band peaking at 238–240 nm might contain a contribution from the X-Pro peptide chromophore in *cis* conformation. This suggestion is compatible with the observation from ^{13}C-NMR of a 14% partition of *cis* conformation of TRF dissolved in water[5].

These CD studies of TRF and of some of its analogues indicate that in dioxane selected conformations occur in the region of the histidine and proline residues which are not affected by the pyroglutamic acid residue. Prolinamide is most likely bonded by one of the carboxamide protons to the carbonyl oxygen of histidine.The imidazole ring is probably linked to a group which cannot be identified by the experiments made. It is possible that the histidine peptide NH group either hydrogen bonds to the imidazole N^{π} atom or to the π-electron system in its vicinity[26–28]. This interaction plays directly or indirectly a role in stabilization of the prolinamide conformation. CPK model building reveals that TRF can adopt such a spatial arrangement without undue constraints. The imidazole ring is close to the proline ring and this mutually restricts their rotations. Substitution of the imidazole ring by a different side-chain can be expected to reduce the stability of prolinamide fixation by hydrogen bonding. If the solvent is able to weaken hydrogen bonds, the prolinamide might become free to rotate. This would explain the CD spectral changes of [Lys^2]-TRF when passing from dioxane to hexafluoro-2-propanol and stresses the role of the solvent in the conformational characteristics of TRF. Since the peptide is small, the cooperation of intramolecular forces to hold together a given conformation is limited and challenged by solvation. Water in particular seems very efficient in this respect, as indicated by the reduced intensity of the TRF negative band at 226 nm when its dioxane solution is hydrated (Fig. 2).

There is no conflict between the conclusions of this work and those presented by other authors using other solvents, such as water or dimethyl sulphoxide[6,7]. Great care is necessary before data obtained under one set of conditions or with TRF analogues can be extrapolated to TRF in a different environment.

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