Solution Conformation of Tuftsin

Annamaria D'Ursi,[‡] Monica Pegna,[§,∥] Pietro Amodeo,[‡] Henriette Molinari,[∥,⊥] Antonio Verdini,[§] Lucia Zetta,[∥] and Piero A. Temussi*[,‡]

Dipartimento di Chimica, Università di Napoli, via Mezzocannone 4, Napoli, Italy, Italfarmaco S.p.A., via dei Lavoratori 54, Cinisello Balsamo, Milano, Italy, Dipartimento di Chimica Organica e Industriale, Università di Milano, via Venezian, Milano, Italy, and I.C.M. del C.N.R., via Ampère 56, 20131 Milano, Italy

Received March 17, 1992; Revised Manuscript Received June 17, 1992

ABSTRACT: Tuftsin, a natural linear tetrapeptide (Thr-Lys-Pro-Arg) of potential antitumor activity, has been studied in DMSO-d_6 solution by 2D NMR spectroscopy. ^{1}H and ^{13}C spectra show the presence of two families of conformations characterized by a trans or cis Lys–Pro bond, respectively. The family of conformers containing the cis peptide bond is a mixture of extended structures as expected for a short linear peptide. On the contrary, the trans isomer appears to be a rigid, folded conformer, as indicated by crucial NOEs and by the exceptionally low temperature coefficient of Arg NH. Analysis of the solution data by means of energy calculations leads to a unique structure, characterized by a Lys-Pro inverse γ-turn.

Tuftsin (Nishioka et al., 1972, 1973a,b) is a linear tetrapeptide (H-Thr-Lys-Pro-Arg-OH) that corresponds to residues 289–292 of leukokinin (Edelman et al., 1969), a cytophilic γ-globulin (Fidalgo & Najjar, 1967) that stimulates the phagocytic activity of the polymorphonuclear cell and the macrophage (Constantopoulos & Najjar, 1972). All stimulatory activity of this protein can be attributed to tuftsin (Najjar, 1974), that is released by proteolytic enzymes after the carrier protein binds to the target cells.

In addition to stimulation of the phagocytic activity (Constantopoulos & Najjar, 1972), tuftsin has several related biological activities; e.g., it is chemotactic (Nishioka et al., 1973b; Lukacs et al., 1984), increases the level of cyclic guanylate (Stabinsky et al., 1980), stimulates the motility of granulocytes and macrophages (Nishioka et al., 1973b), and promotes bacterial killing properties (Martinez & Winternitz, 1983) and the tumoricidal activity of phagocytic cells (Nishioka, 1979; Najjar et al., 1983).

Numerous SAR[1] studies (Nishioka et al., 1973b; Fridkin et al., 1977; Hisatsune et al., 1978; Konopinska et al., 1978, 1979; Konopinska, 1978; Fridkin & Gottlieb, 1981; Siemion & Konopinska, 1981) have shown that the activity of tuftsin is very sensitive to constitution; all substitutions have led to analogues with lower activity. The conformation of tuftsin has been studied both experimentally by means of several spectroscopic techniques, i.e., NMR (Siemion et al., 1980; Blumenstein et al., 1979; Sekacis et al., 1979), IR (Suchardа-Sobczyk et al., 1979), and CD (Siemion et al., 1980, 1983), and theoretically by means of internal energy calculations (Nikiforovich, 1978; Fitzwater et al., 1978). Structural proposals made by different authors differ considerably, even for studies performed in the same solvent. For instance, in aqueous solution, Blumenstein et al. (1979) interpreted ^{1}H and ^{13}C NMR spectra on the basis of a disordered conformation, whereas Siemion et al. (1980, 1983) propose a type III β-turn on the basis of ^{13}C NMR and CD spectra.

The situation in DMSO-d_6 solution is even more puzzling since, in spite of clear indications in favor of a folded structure, the authors did not postulate any definite structural model (Blumenstein et al., 1979). In particular, the ^{1}H data in DMSO-d_6 showed a temperature coefficient of the resonance of the Arg NH proton very close to zero. This value, quite outstanding for a short linear peptide, reveals the presence of a thermally stable hydrogen bond that, as confirmed by our study at a markedly lower concentration (vide infra), is consistent with a folded conformation. In the quoted paper (Blumenstein et al., 1979) no use was made of this indication because the only structure considered, a β-turn, was judged unlikely owing to the unusual presence of Pro in the $i + 2$ position of the turn, but also because the quality of the spectra did not allow the measurement of diagnostically valuable NOEs, the only NMR data on which a detailed structural interpretation can be reliably based. In addition, it must be noted that experimental shortcomings prevented even the detection of two isomers, linked to the cis–trans isomerism of the Lys–Pro bond, and consequently, structural proposals could not be based on a reliable assignment of the conformation of this crucial peptide bond (Blumenstein et al., 1979).

Theoretical analysis of the conformational space of tuftsin (Fitzwater et al., 1978), performed prior to the quoted NMR work (Blumenstein et al., 1979), found that the area of global minimum of the energy surface is characterized by many individual conformations of comparable energy, globally referred to as "hairpins with split ends".

Here we present a detailed conformational analysis of tuftsin based on ^{1}H and ^{13}C NMR data in DMSO-d_6 solution and on internal energy empirical calculations.

MATERIALS AND METHODS

Tuftsin was purchased from Bachem (Switzerland) and used without further purification. Five-millimeter tubes (Wilmad, Buena, NJ) and DMSO-d_6 (99.98% isotopical purity, Aldrich, Milwakee, WI) were used for NMR spectra.

NMR spectra were recorded on AM 400 and AM 500 Bruker spectrometers and on Unity 400 MHz Varian spec-

[‡] Università di Napoli.
[§] Italfarmaco S.p.A.
[∥] I.C.M. del C.N.R.
[⊥] Università di Milano.

[1] Abbreviations: BFGS, Broyden–Fletcher–Goldfarb–Shanno algorithm; CD, circular dichroism; DMSO-d_6, perdeuteriodimethyl sulfoxide; DQF-COSY, double-quantum-filtered correlation spectroscopy; EM, energy-minimization calculations; IR, infrared spectroscopy; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; SAR, structure–activity relationship; TMS, tetramethylsilane; TPPI, time proportional phase increment.

0006-2960/92/0431-9581$03.00/0

Table I: Comparison of NOE Constraints and Calculated Distances for the Four Best Conformers of the Conformational Search (I–IV)

NOE	exp range	conf I	conf II	conf III	conf IV
ArgNH-ProCH$_\alpha$	2.3–2.9	2.24	2.25	2.54	2.55
LysCH$_\alpha$-ProCH$_\delta$	2.3–3.4	2.27	2.98	2.36	2.37
LysCH$_\alpha$-ProCH$_{\delta'}$	2.3–3.4	2.51	2.39	2.41	2.41
ThrCH$_\alpha$-LysNH	3.5–4.6	3.57	3.47	3.60	3.61
LysNH-LysCH$_\alpha$	2.9–3.6	2.84	2.87	2.85	2.91
ArgNH-ArgCH$_\alpha$	2.5–3.2	2.27	2.24	2.94	2.94

trometers. The peptide concentration was 4 mM in DMSO-d_6. All chemical shifts, in parts per millions (ppm), are referred to internal tetramethylsilane (TMS). Proton one-dimensional (1D) spectra have been acquired using typically 16–32 scans with 16K data size; 32K and 5000–15000 scans have been used for ^{13}C experiments. For the two-dimensional (2D) experiments, both homo- and heterocorrelated, pulse programs of the standard Bruker software library were used. All 2D experiments, except the heterocorrelated ones, have been acquired in the phase-sensitive mode by use of the time proportional phase increment (TPPI).

NOEs were measured at different mixing times, in the range 100–400 ms, as ratios of cross peak volumes to those of the corresponding diagonal peaks. The possible presence of spin diffusion was checked by plotting NOE/τ_m vs τ_m, as suggested by Majumdar and Hosur (1990). A negative slope shows absence of spin diffusion, whereas a positive slope is a sign of spin diffusion; extrapolation to zero τ_m yields a value of the relaxation parameter (σ) that represents a better approximation than traditional buildup, even in the presence of (moderate) spin diffusion. The NOESY with a τ_m of 200 ms was used to calculate NOE constraints. The ratios can be translated into interproton distances by the method of Esposito & Pastore (1988), provided it is possible to calibrate one of the NOEs with a known distance. Unfortunately, it is not possible to use any of the observed NOEs as internal standard to scale the other effects. However, it is possible to use the two cross peaks between K_α and the two protons of the γ-CH_2 of P to find at least a relative scale of all NOEs. The ratio between the two effects, $R = K_\alpha/P_\delta{:}K_\alpha/P_{\delta'}$, depends only on the ψ angle of the K–P bond; the experimental ratio is 2.83. By exploring all values of R as a function of ψ, it is possible to calculate the theoretical ratios (independent from the correlation time): it is clear that a ratio of 2.83 can be reproduced only for the ranges 50–60°, 110–120°, −130 to −120°, and −80 to −70°, but the ranges −130 to −120° and −80 to −70° can be excluded since they correspond to large distances that would yield very low absolute values, inconsistent with the intense NOEs observed. A final choice between the two allowed ranges can be made through a systematic search of all conformational space (vide infra), but the values of the interproton distances indicated by the two ranges, ca. 2.4 and 2.9 Å for K_α/P_δ and $K_\alpha/P_{\delta'}$, respectively, are sufficient to calculate a likely range for each of the interproton distances corresponding to observed NOEs. The relevant constraints are summarized in Table I.

Conformational searches were performed by systematic variation of the selected dihedral angles (see Results), with a 30° resolution, using the search module of SYBYL package (version 5.3). The acceptance criteria were based on interatomic distances between pairs of nonbonded atoms, plus, in restrained search only, six constraints derived from NOE data (Table I). In particular, a conformation was rejected whenever it presented one or more interatomic distances below a threshold represented by the scaled van der Waals distances, with scaling factors of 0.85, 0.75, and 0.70, respectively, for

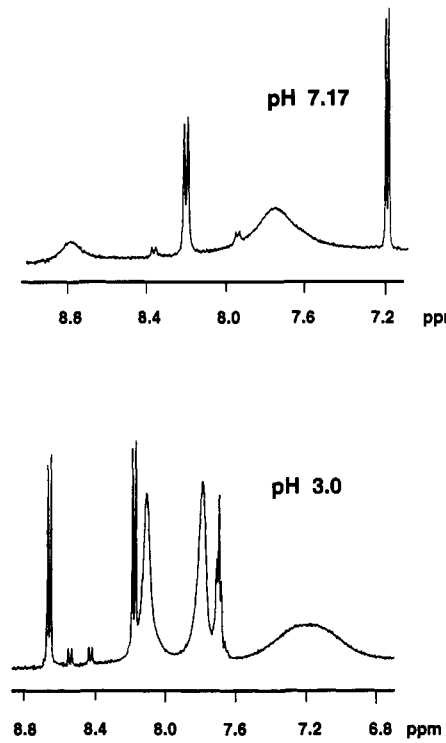

FIGURE 1: Comparison of the low-field region of the 400-MHz ^1H NMR spectra of tuftsin in DMSO-d_6 at 298 K, corresponding at two representative ionization conditions (pH 7.17 and pH 3.0). Both samples are 4 mM.

distal (1–5 or more), vicinal (1–4), and H-bond interactions. NOE constraints were introduced by accepting only those conformations in which the six considered distances were included in the ranges reported in Table I.

As the 5.3 version of SYBYL does not allow either energy minimization (EM) or the use of the AMBER force field during the search, a command procedure, written in the SYBYL SPL language, has been developed in order to remove the main sterical strain, by performing 20 unrestrained EM steps, on each conformation obtained in the search. In this way the reliability of a selection based on energetic thresholds is considerably increased.

The final unrestrained EMs in each series of calculations have been performed according to the following scheme: the all-atom (Weiner et al., 1986) parametrizations of the AMBER force field were used in a series of EM calculations. The computational procedure can be divided into two steps: (i) an EM calculation is performed, using a quasi-Newton method, the Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm (Press et al., 1986), stopping when the gradient norm is 10^{-3} or less and (ii) a final refinement is obtained by a full Newton–Raphson minimization, with a convergence criterium on the gradient norm of 10^{-6} or less.

Solvent accessibility has been calculated by means of the program GEPOL (Silla et al., 1990).

RESULTS

NMR. The proton spectrum of tuftsin in DMSO-d_6 is dependent on the ionization state of the dissolved peptide; accordingly, several samples, corresponding to different ionization states, were prepared by titration in aqueous solution followed by lyophilization prior to dissolution in DMSO-d_6. Figure 1 shows the comparison of the 400-MHz ^1H spectra of tuftsin at two representative ionization conditions; the spectrum changes very little for samples corresponding to pHs

Table II: Proton Chemical Shifts (δ) of a Tuftsin Sample Corresponding to pH 7.17 and Temperature Coefficients of Amidic Protons in DMSO-d_6

residue	NH	α-CH	β-CH_2	others	δ-NH/K
Thr		3.06	3.84	γ-CH_3 1.05	
thr		3.09	3.96	γ-CH_3 1.11	
Lys	8.20	4.64	1.83/1.81	γ-CH_2 1.42	−4.6
				δ-CH_2 1.55	
				ϵ-CH_2 2.80	
lys	8.36	4.68	1.64	γ-CH_2 1.36	−6.1
				δ-CH_2 1.55	
				ϵ-CH_2 2.76	
Pro		4.46	2.08	γ-CH_2 1.98	
			1.92	δ-CH_2 3.78	
				δ'-CH_2 3.71	
pro		4.34	2.28	γ-CH_2 1.88	
			2.05	δ-CH_2 3.52	
				δ'-CH_2 3.48	
Arg	7.19	3.90	1.73	γ-CH_2 1.45	0
			1.59	δ-CH_2 3.11	
arg	7.92	4.10	1.70		−7.3

(in aqueous solution) from 7.17 to 5.0, whereas dramatic changes take place at lower pH values. Spectral changes observed at low pHs reflect disruption of the well-defined conformational state observed in the 5–7.17 pH range, possibly owing to the crucial role of the terminal carboxyl group in protecting the Arg NH (vide infra). Accordingly, all detailed conformational data were collected on samples corresponding to pH 7.17.

Assignment of proton resonances is straightforward since, owing to the simple chemical constitution, there is no need for sequential assignment. The only problem was posed by the partial superposition of the resonances of the side chains of Lys and Arg, but a combination of DQF-COSY and TOCSY spectra gave an unambiguous answer, confirming the previous assignment of the two NH resonances (Blumenstein et al., 1979), based on the indirect evidence of titration behavior. All relevant proton chemical shift data are summarized in Table II along with NH temperature coefficients.

There are two families of conformations, since most resonances are split into a major and a minor component as expected from a cis–trans isomerism around the Lys–Pro bond. It can be noted (from Figure 1) that the separation of the two components of each resonance is much lower at pH 3. The origin of the satellite resonances was confirmed by exchange cross peaks observed in ROESY and NOESY spectra; Figure 2 shows a portion of the NOESY spectrum of tuftsin containing the prominent cross peaks between cis and trans resonances and the diagnostic cross peak between Lys α-CH and Pro δ-CH_2 protons. Assignment of the two components to cis and trans isomers is crucial for the subsequent structural interpretation; accordingly it is not possible to rely on the often used correlation that attributes the larger component to the trans isomer on the basis of its (generally) greater stability. We found, however, that indeed the larger component corresponds to the trans isomer; this assignment is based on the presence of a diagnostic cross peak between the Lys α-CH and the Pro δ-CH_2 resonances and on the typical values of the resonances of the ring carbons of Pro. Figure 3 shows the portion of the reverse heterocorrelated ^1H–^{13}C spectrum containing the relevant Pro carbon resonances. Table III summarizes all carbon chemical shift data along with typical literature values (Wüthrich & Grathwohl, 1974) for the ring carbons of Pro in trans and cis peptide bonds.

The proton data contain two unusual features that can be attributed to conformational effects. Splitting of the reso-

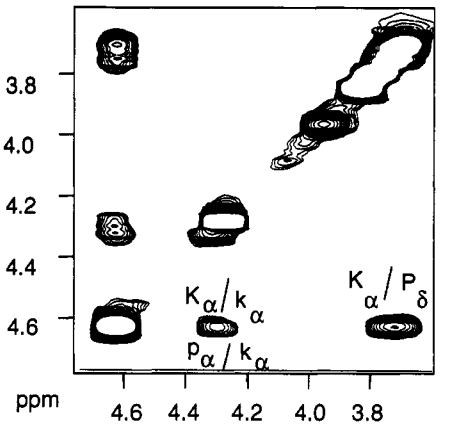

FIGURE 2: Partial 400-MHz NOESY spectrum of tuftsin (aliphatic region) showing exchange cross peaks between cis and trans resonances and the diagnostic cross peaks between Lys α-CH and Pro δ-CH_2 protons: DMSO-d_6 solution at 298 K.

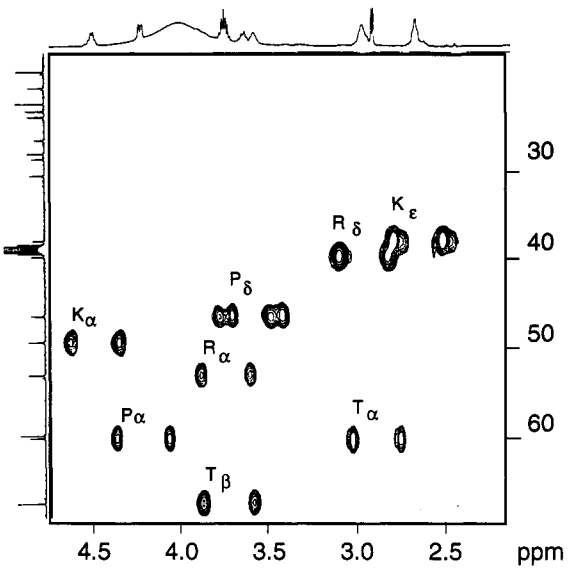

FIGURE 3: 500-MHz partial reverse heterocorrelated ^1H–^{13}C spectrum of tuftsin: expanded region containing the relevant Pro carbon resonances: 6 mM DMSO-d_6 solution at 298 K.

Table III: Carbon Chemical Shifts of Tuftsin in DMSO-d_6 with Typical Literature Values for the Ring Carbons of Pro in Trans and Cis Bonds

residue	α-C	β-C	γ-C	others
Thr	60.7	~6.8	20.2	
thr	60.1	67.1	20.4	
Lys	50.0	31.6	21.9	δ 27.7
				ϵ 38.8
lys	50.5	32.1	22.1	δ 28.0
Pro	60.5	29.2	24.5	δ 47.1[a]
	59.6	26.7	24.3	δ 46.0[b]
pro	59.1	32.6	21.9	δ 47.1[a]
	60.5	32.1	22.2	δ 46.8[b]
Arg	53.6	29.2	25.1	δ 40.5
arg	53.9	28.0	25.3	

[a] Observed. [b] Wüthrich, K., & Grathwohl, Ch., 1974.

nances due to the cis–trans isomerism is not confined to Lys protons, i.e., the residue preceding Pro, as is typical for peptides, containing an Xaa–Pro bond, in a random coil conformation. Rather, practically all resonances are affected; in particular it is worth noticing that the NH resonance of Arg, i.e., the residue following Pro, shows a cis–trans separation greater than that of Lys NH. Such a behavior is only possible if at least one of the two families of isomeric molecules contains

a stable, folded conformation that can transmit the effect of the Xaa–Pro bond isomerism. The fact that the temperature coefficient of the Arg NH is nearly zero for trans molecules indicates that these molecules are characterized by a strong, thermally stable hydrogen bond. It must be noted that the occurrence of such a low temperature coefficient in a linear peptide containing only four residues is quite unusual. On the other hand, the corresponding cis resonance has a coefficient of −7.3 ppb/K, a value typical of a solvent exposed NH.

As mentioned in the introduction, the low value of the coefficient of Arg NH had already been reported in the literature (Blumenstein et al., 1979), albeit with the uncertainty about its assignment to the cis or trans isomer. No structural model was proposed on this basis since the only possibility considered, i.e., a β-turn, was judged somewhat inconsistent with the presence in the $i + 2$ position of the turn of a trans Pro residue (Chandrasekaran et al., 1973; Chou & Fasman, 1978). The availability of more advanced instrumentation allows us to test the feasibility of a β-turn, also in comparison with other folded structures energetically consistent with the primary structure of tuftsin.

To this end it is crucial to obtain diagnostically valuable NOEs. In fact, for a peptide this size, the very detection of NOEs at high field is already a strong indication of the presence of a fairly rigid structure since, in general, the unfavorable correlation time of short peptides combined with conformational mobility leads to negligible NOEs (Morris, 1986; Temussi et al., 1989). In the NOESY spectrum of tuftsin it is possible to observe numerous intrachain cross peaks along with the mentioned exchange peaks between corresponding cis and trans resonances (see Table I). Moreover, there are two prominent interresidue peaks that suggest a likely structural interpretation of all NMR data, i.e., that between the α-CH of Lys and the δ-CH_2 of Pro and that between the α-CH of Pro and the NH of Arg. They are not consistent with either β-turns or a hairpin with split ends but point to a γ-turn structure centered on the Lys-Pro moiety.

Conformational Analysis. The combination of a few diagnostically significant NOEs and of the very low temperature coefficient of Arg NH chemical shift might be sufficient to propose a likely folded conformation. Notwithstanding, we have tried to perform a complete search of the conformational space available to tuftsin. A similar search is generally prohibitive even for fairly small peptides but can become feasible in the case of tuftsin owing to the limitations inherent in the constitution and, eventually, by taking advantage of the constraints suggested by the NMR data. In fact, similar energy computations of tuftsin have already been performed in the past (Fitzwater et al., 1978), but these authors could not take advantage of NMR data. The first step (I) in our search was completely unrestricted; that is, we allowed a complete variation of all internal rotation angles. The criteria for conformation selection are described in Materials and Methods. This totally unconstrained search leads to the unwieldly number of over 500 000 conformers.

A second exploratory search (II) allowed only ψ_1, χ_1, ϕ_2, ψ_2, ψ_3, ϕ_4, ψ_4 to vary completely, whereas all other χs were kept fixed, with the conformations of the side chains of Lys and Arg fully extended. This choice limited the accessibility of conformational space severely, leading to "only" 18 208 conformations. Large as this number may seem, the criterion of fully extended chains for the two long-chain residues can still be too crude an approximation since it is likely that parts of conformational space are artificially excluded.

Table IV: Backbone Internal Coordinates of the Best Conformers of Thr[1]-Lys[2]-Pro[3]-Arg[4], Derived from the Search and EM Procedures with $\epsilon = 1$ (I, II) and $\epsilon = 10R$ (III, IV)[a]

	I	II	III	IV
ψ_1	−83	−63	−71	−63
ϕ_2	−54	−45	−73	−156
ψ_2	135	169	149	137
ϕ_3	−79	−59	−82	−78
ψ_3	143	88	51	66
ϕ_4	48	73	−146	−146
solvent access (Å²)	14	7	0.1	6

[a] The last row shows the solvent accessibility of Arg NH in Å².

That this was indeed the case was proved by a similar search (III) performed on a model tuftsin: Thr-Ala-Pro-Ala (henceforth dubbed ATUF). A complete search on the same angles of search II led, in this case, to 77 890 conformations, i.e., a number much larger than that found in search II. Thus, it is clear that search II was largely influenced by the rigidity of Lys and Arg side chains. The only way to narrow the spread of accessible conformers is to introduce further constraints. Introduction of six crucial NOE constraints (Table I) has a dramatic effect on the number of conformers found for ATUF, which drop from 77 890 to 1796. Introduction of an energy-minimization criterion (EM performed with $\epsilon = 1$), albeit with a generous threshold of 40 kcal/mol, further reduces the number of conformers to 151.

Close inspection of these conformers reveals that the actual number may be further reduced if one takes into account that there are families on conformations differing only in the values of the C-terminal ϕ_4, ψ_4. By selecting families with similar ϕ_4, ψ_4 values and keeping only the lowest energy member of the family as representative of the whole family, the total number of acceptable conformers reduces to a mere 14 conformers. These conformers can be arranged in pairs that differ only by the value of χ_1; by choosing the lowest energy of each pair, they are immediately reduced to 7. These structures have been selected as starting models for unrestrained EM calculations on the whole tuftsin molecule, choosing an extended conformation for the side chains of Lys and Arg. Full geometry energy minimization of these conformers yields two most probable global conformations, whose internal backbone coordinates are listed in Table IV.

The search procedure based on EM was repeated with $\epsilon = 10R$ to test the relevance of electrostatic interactions in the stabilization of structures containing the head-to-tail salt bridge. The damping of electrostatic interactions flattens considerably the potential energy surface of ATUF. In fact, now the energies of the 1796 conformations cover a range of about 12 kcal mol^{-1} only. In this case, introduction of an energy-minimization criterion (EM performed with $\epsilon = 1$), with a threshold of 5 kcal/mol, reduces the number of conformers to the still considerable number of 1008. However, the barriers among different conformers are also reduced, so the partial EM procedure and the elimination of similar conformations (see above) reveal that the actual number may be drastically reduced if one takes into account that there are families of conformations differing only in the values of the C-terminal ϕ_4, ψ_4. By selecting families with similar ϕ_4, ψ_4 values and keeping only the lowest energy member of the family as representative of the whole family, the total number of acceptable conformers reduces to 19 conformers, which, after full unrestrained EM, reduce to six conformers. These structures of ATUF have been used as starting conformations for full EM on the complete tuftisin molecule, by introduction of all side chains, arranged in the most favorable conformation

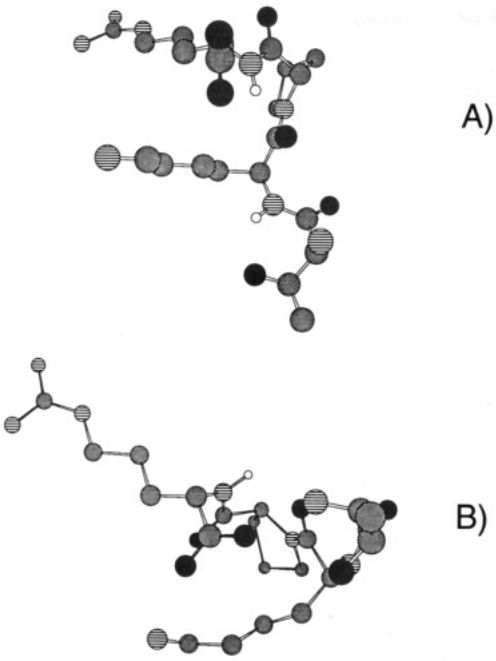

FIGURE 4: Molecular models of conformer III (A), the only conformer, among the energy minima, that shows a nearly complete solvent inaccessibility of Arg NH and of conformer II (B), the best conformer among the energy minima calculated with $\epsilon = 1$. Only amidic hydrogens are shown.

for each backbone structure. When only different backbone conformations are considered, the six structures reduce to two, also listed in Table IV.

The conformational space of these conformers was further examined for the arrangement of the side chains of Arg and Lys, but only conformers with pairs of χ_1 for Lys and Arg respectively of 180°, –60° and 60°, 60° could be excluded, whereas all other arrangements are within an energy range of 2 kcal/mol. It is not possible, at this stage, to discriminate among the four preferred structures on the basis of NOE constraints or of potential energy. In fact, all structures of Table IV fulfill reasonably well the requirements of the NOE constraints in spite of the fact that they are derived from unrestrained minimizations (see Table I), and as far as energy is concerned, it is important to recall that solvation effects and entropic contributions can be decisive in the choice of "the best conformer". A further, more restrictive criterion, is represented by the accessibility of the NH proton of Arg, since it is difficult to account for a zero temperature coefficient, in a tetrapeptide, by the only requirement of an internal hydrogen bond in cases of sizeable solvent accessibility. Accordingly, we calculated the area of Arg NH accessible to solvent molecules for all conformers of Table IV (Silla et al., 1990). It is clear that only conformer III is completely consistent with this very strict requirement. The solvent accessible area for Arg NH in this conformer, 0.09 Å^2, should be compared with a figure of 21 Å^2 for a completely exposed NH, as calculated for *N*-methylacetamide. The very limited solvent accessibility is paralleled by the formation, by this hydrogen, of a strong hydrogen bond with Lys CO and of a second hydrogen bond with the terminal carboxyl group. The pair of χ_1s, for Lys and Arg respectively is 60°, –60° for the most favorable arrangement of the side chains with respect to solvent accessibility of Arg NH (0.09 Å^2); the average accessibility for all allowed arrangements is 0.76 Å^2. The different accessibility of Arg NH can be appreciated also from a comparison of the two models of Figure 4: Figure 4A shows the molecular model of conformer III, whereas Figure 4B shows the best conformer calculated at $\epsilon = 1$, i.e., conformer II. It is clear that the introduction of a very limited number of constraints, i.e., a trans Xaa–Pro bond and the relevant NOEs between the α-CH of Xaa and the δ-CH_2 of Pro and between the α-CH of Pro and the NH of the following residue, is sufficient to restrict enormously the number of accessible conformations.

A similar conformation, conformer FCE(I) in their notation, can be found among the 162 trans conformers used by Fitzwater et al. (1978) to describe the conformational state of tuftsin. However, according to Fitzwater et al. (1978) conformer FCE(I) was one of the *minor* conformers of the conformational mixture since "...the C_7 hydrogen bond was absent in the lowest energy trans conformations".

It is possible to conclude that the structure of tuftsin found in DMSO solution is consistent with all NMR data and represents well the predominant conformational state in solution for the family of molecules containing a trans Lys–Pro bond. The preferred conformation in DMSO solution is not necessarily the bioactive conformation of tuftsin, since such a small molecule may retain considerable flexibility to adapt to the receptor. It is in order to emphasize, however, that this structure represents the first reliable, stable conformation found experimentally for this peptide and may be considered a good lead for a more rational drug design.

ACKNOWLEDGMENT

A.D. thanks Italfarmaco for a 2-year fellowship. P.A. thanks Dr. J. L. Pascual-Ahuir for providing us with a copy of the program GEPOL.

REFERENCES

Blumenstein, M., Layne, P. P., & Najjar, V. A. (1979) *Biochemistry 18*, 5247–5253.
Chandrasekaran, R., Lakshminarayan, A. V., Pandya, U. V., & Ramachandran, G. N. (1973) *Biochim. Biophys. Acta 303*, 14–27.
Chou, P. Y., & Fasman, G. D. (1978) *Am. Rev. Biochem. 47*, 251–273.
Constantopoulos, A., & Najjar, V. A. (1972) *Cytobios 6*, 97–100.
Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauer, U., & Waxdal, M. J. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 78–85.
Esposito, G., & Pastore, A. (1988) *J. Magn. Reson. 76*, 331–336.
Fidalgo, B. V., & Najjar, V. A. (1967) *Biochemistry 6*, 3386–3392.
Fitzwater, S., Hodes, Z. I., & Scheraga, H. A. (1978) *Macromolecules 11*, 805–811.
Fridkin, M., & Gottlieb, P. (1981) *Mol. Cell Biochem. 41*, 73–78.
Fridkin, M., Stabinsky, Y., Zakuth, V., & Spirer, Z. (1977) *Biochim. Biophys. Acta 496*, 203–211.
Hisatsune, K., Kobayashi, K., Nozaki, S., & Muramatsu, I. (1978) *Microbiol. Immunol. 22*, 581–584.
Konopinska, D. (1978) *Pol. J. Chem. 52*, 953–957.
Konopinska, D., Siemion, I. Z., Szymaniec, S., & Slopek, S. (1978) *Pol. J. Chem. 52*, 573–580.
Konopinska, D., Siemion, I. Z., Szymaniec, S., & Slopek, S. (1979) *Pol. J. Chem. 53*, 343–351.
Lukacs, K., Szabo, G., Sonkoly, I., Vegh, E., Gacs, J., Szegedi, G., & Szekerke, M. (1984) *Immunopharmacology 7*, 171–178.
Majumdar, A., & Hosur, R. V. (1990) *J. Magn. Reson. 88*, 284–290.
Martinez, J., & Winternitz, F. (1983) *Ann. N.Y. Acad. Sci. 419*, 23–31.
Morris, G. A. (1986) *Magn. Reson. Chem. 24*, 371–403.

Najjar, V. A., Lineham, L., & Nonopinska, D. (1983) *Ann. N.Y. Acad. Sci. 419*, 261–267.
Nikiforovich, G. V. (1978) *Bioorg. Khim. 4*, 1427–1430.
Nishioka, K. (1979) *Br. J. Cancer 39*, 342–345.
Nishioka, K., Constantopoulos, A., Satoh, P. S., & Najjar, V. A. (1972) *Biochim. Biophys. Res. Commun. 47*, 172–179.
Nishioka, K., Constantopoulos, A., Satoh, P. S., Mitchell, W. M., & Najjar, V. A. (1973a) *Biochim. Biophys. Acta 310*, 217–229.
Nishioka, K., Satoh, P. S., Constantopoulos, A., & Najjar, V. A. (1973b) *Biochim. Biophys. Acta 310*, 230–237.
Press, W. A., Flannery, B. P., Teukolsky, S. A., & Vetterling, W. T. (1986) *Numerical recipes, the art of scientific computing*, pp 307–312, Cambridge University Press, Cambridge.
Siemion, I. Z., & Konopinska, D. (1981) *Mol. Cell Biochem. 41*, 99–111.
Siemion, I. Z., Lisowski, M., & Sobczyk, K. (1983) *Ann. N.Y. Acad. Sci. 419*, 56–63.
Siemion, I. Z., Lisowski, M., Konopinska, D., & Nawrocka, E. (1980) *Eur. J. Biochem. 112*, 339–343.
Silla, E., Villar, F., Nilsson, O., Pascual-Ahuir, J. L., & Tapia, O. (1990) *J. Mol. Graphics 8*, 168–172.
Stabinsky, Y., Bar-Shavit, Z., Fridkin, M., & Goldman, R. (1980) *Mol. Cell. Biochem. 30*, 71–77.
Temussi, P. A., Picone, D., Castiglione-Morelli, M. A., Motta, A., & Tancredi, T. (1989) *Biopolymers 28*, 91–107.
Weiner, S. J., Kollman, P. A., Nguyen, D. T., & Case, D. A. (1986) *J. Comput. Chem. 7*, 230–252.
Wüthrich, K., & Grathwohl, Ch. (1974) *FEBS Lett. 43*, 337–340.

Registry No. Tuftsin, 9063-57-4.