Conformation of a Heptapeptide Substrate Bound to Protein Farnesyltransferase†

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ABSTRACT: Protein farnesyltransferase catalyzes isoprenylation of the cysteine four residues from the C-terminus of several proteins including $p21^{ras}$. Farnesylation is required for the transforming activity of Ras, and many efforts are underway to develop inhibitors of farnesyltransferase. We have used nuclear magnetic resonance spectroscopy to determine the farnesyltransferase-bound conformation of a heptapeptide substrate, KTKCVFM, which competes for the modification of $p21^{Ha\text{-}ras}$ in an *in vitro* assay. Analysis of transferred nuclear Overhauser effects reveals that the CVFM sequence of the peptide substrate is directly involved in binding to the enzyme and adopts a type I β-turn conformation in the bound state. The present structural information should aid in the design of more effective inhibitors of the enzyme and in understanding the nature of the peptide binding site.

Posttranslational modification of Ras and several other intracellular proteins begins with a covalent attachment of an isoprenoid group via a thioether linkage to a cysteine residue four amino acids from the C-terminus (Clarke, 1992). This modification is followed by proteolysis of the last three residues and then carboxymethylation of the now C-terminal cysteine. Proteins that are known to be modified by attachment of a farnesyl group (C15) have a C-terminal CAAX motif, where C is cysteine, A is any aliphatic residue, and X is serine, alanine, or methionine. Another transferase recognizes a CAAX motif in which X is usually a leucine and modifies the cysteine with a geranylgeranyl (C20) group. Both enzymes function as heterodimers and share a common α subunit (Seabra et al., 1991). Their β subunits differ and are believed to confer specificity in protein substrate binding (Chen et al., 1991). Although there have been many studies characterizing isoprenyl transferases, to date there is no detailed structural information on these enzymes. Since the transforming activity of oncogenic Ras proteins is dependent on their farnesylation, inhibitors of isoprenyl transferases are of great interest as potential cancer therapeutics.

Previous studies revealed that small peptides such as the tetrapeptide CVIM compete with protein substrates and can be farnesylated by farnesyltransferase (Reiss et al., 1990). In a study of the sequence requirements for peptide recognition by the enzyme, we found that CVFM was the most potent inhibitor among 42 CXXX variants (Reiss et al., 1991). Interestingly, most sequence variants were substrates and thus were farnesylated by the enzyme, but substitution of penicillamine (Pen)[1] for the cysteine or introduction of phenylalanine as the penultimate residue (*e.g.*, CVFM, CIFM, PenVFM, and PenVIM) prevented farnesylation of the peptide and allowed it to act as a true inhibitor (Goldstein et al., 1991). However, N-terminal modifications of CVFM or removal of the cysteine amino group reversed this effect, and the peptide became a substrate again (Goldstein et al., 1991; Brown et al., 1992).

To build on these studies in the design of further inhibitors and to enhance our understanding of the mechanism of the farnesyltransferase, we have determined the conformation of a peptide substrate bound to the enzyme. This analysis was made possible by the development of an efficient expression system for the protein farnesyltransferase which has provided ample quantities for biophysical study (Reiss et al., personal communication; James et al., 1993). The technique of transferred nuclear Overhauser effect (trNOE) NMR spectroscopy (Clore & Gronenborn, 1982, 1983) is ideally suited to study the conformation of a low molecular weight ligand bound to an enzyme. This method relies on the large difference in correlation time between the free ligand and the ligand–enzyme complex. In the rapidly tumbling free ligand, interproton magnetization transfer is inefficient and NOEs are near zero. When the ligand in its bound state tumbles with the larger molecule, there is efficient transfer of magnetization between protons and large NOEs can develop, in a distance-dependent manner. In a fast-exchange situation, these NOEs are transferred into the free ligand population where they persist for a time governed by the relaxation time of the free ligand protons. Hence, observation of the interproton NOEs of the free ligand reveals the distance relationships of the bound ligand. Because the enzyme is present in substoichiometric amounts and its proton signals are very broad, its NMR resonances are generally unobserved.

We chose to use a peptide substrate in these studies, KTKCVFM, which corresponds to the last seven residues of the $p21^{Ki\text{-}rasB}$ protein, with the penultimate isoleucine of the protein replaced by a phenylalanine. This peptide inhibits farnesylation of $p21^{Ha\text{-}ras}$ in our standard assay (Reiss et al., 1991) with an apparent K_i of 4.5 μM. A tetrapeptide corresponding to the same C-terminal four residues (CVFM) is a more potent inhibitor (apparent K_i = 0.06 μM; Brown et al., 1992) but could not be used in the present work due to its limited aqueous solubility. Our trNOE results show that the heptapeptide binds with the terminal tetrapeptide forming a type I β turn. Additionally, these residues are the most strongly influenced by the binding interaction with farnesyltransferase. This result is entirely consistent with the recent

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[1] Abbreviations: DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; Pen, penicillamine; TOCSY, two-dimensional total correlation spectroscopy; trNOE, transferred NOE.

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report of a potent peptidomimetic inhibitor of farnesyltransferase based on the incorporation of CVFM into a turn mimic (James et al., 1993). Our NMR results should prove useful in design of future inhibitors of farnesyltransferase.

MATERIALS AND METHODS

Peptide Synthesis. The heptapeptides were synthesized either on a Milligen 9050 synthesizer using 9-fluorenylmethyloxycarbonyl chemistry or on an Applied Biosystems 430A synthesizer using *tert*-butyloxycarbonyl chemistry and were purified by reverse-phase high-pressure liquid chromatography (HPLC) on a C18 Vydac stationary phase with an acidified water/acetonitrile gradient (0.1% trifluoroacetic acid). Peptide purity was checked by analytical HPLC and amino acid analysis, and mass spectrometry was used to confirm the peptide identity by checking molecular weights.

Sample Preparation. NMR samples contained 4 mM peptide and 45 μM farnesyltransferase at pH 6 in a solution of 20 mM KCl, 50 μM $ZnCl_2$, 3 mM $MgCl_2$, 20 mM deuterated Tris, 20 mM deuterated malonate (Cambridge Isotope Laboratories, Woburn, MA), 0.3 mM nonionic detergent NP40 (Sigma), 3 mM DTT, and 10% D_2O. The sample was prepared by addition of a concentrated protein solution to the peptide solution. Handling of the protein was critical to the success of the experiment: A stock solution of farnesyltransferase (5–7 mL, 1–3 mg/mL), which had been stored at −70 °C as the active heterodimer complex, was concentrated by ultrafiltration using a Centricon-30 tube (Amicon) and spinning at 6000 rpm at 4 °C. Maintaining the final volume above 0.3 mL minimized the likelihood of enzyme precipitation. The sample was then washed three times with 1.5 mL of the deuterated buffer solution used for NMR (as described above). The final volume of enzyme solution was approximately 100 μL. Also of importance in the outcome of the trNOE experiment was the pH: Exposing the farnesyltransferase to a pH ≤5.9 led to precipitation. On the other hand, if the solution pH were significantly higher than 6.1, NH resonances of the peptide could not be observed due to exchange broadening.

Nuclear Magnetic Resonance. Two-dimensional spectra were obtained at 15 °C on a 500-MHz Varian VXR500 spectrometer in the phase-sensitive mode using the hypercomplex method (States et al., 1982). Data sets consisted of 2 × 256 free induction decays of 1024 complex points each. A 6000-Hz spectral width was used in both dimensions. For NOESY spectra, the mixing times used were 150 and 300 ms. Water suppression was accomplished using a jump and return pulse sequence (Plateau & Guéron, 1982), and additional elimination of the water resonance was performed by deconvolution of the time–domain data (Marion et al., 1989). For TOCSY spectra, a 65-ms mixing time was used, and the water resonance was removed by presaturation. The data were processed using the program Felix (Biosym Technologies, Inc., San Diego, CA 92121). Gaussian and sine-bell (60° shifted) apodizations were used in the t_2 and t_1 dimensions, respectively. In data sets used to calculate volume integrals, a 3-Hz line broadening was used along the t_2 dimension, and a 90° shifted sine bell was used along both the t_1 and the t_2 dimensions. Polynomial baseline correction along the t_2 dimension was carried out before Fourier transformation along t_1. The data were zero-filled in the t_1 dimension to yield matrices consisting of 1024 × 1024 real data points.

Structural Modeling. Molecular dynamics simulations and energy minimizations were performed with the program Discover (Biosym Technologies, Inc.) on a Silicon Graphics 4D/25 Personal Iris workstation. The simulations were carried out *in vacuo* at 300 K using an integration step of 1 fs. Energy minimization was performed on instantaneous structures taken at 2-ps intervals along the trajectories. Selected interproton distances were restrained to be between 2.4 and 2.9 Å using a biharmonic potential that penalized deviations from the distance bounds indicated with a force constant of 30 kcal/(Å² mol).

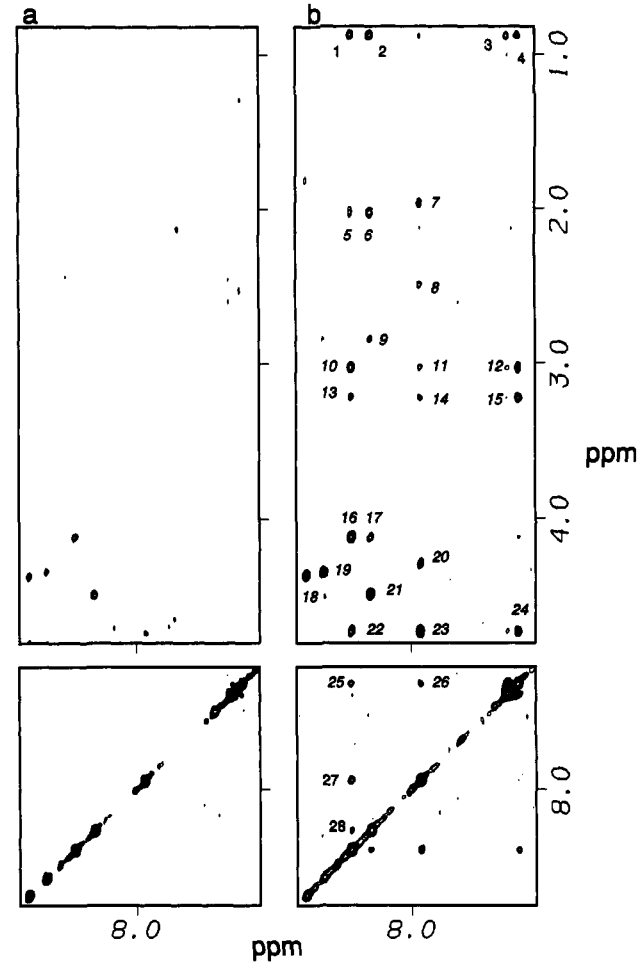

FIGURE 1: Contour plots corresponding to the amide/amide and amide/aliphatic regions of two-dimensional NOE spectra of the peptide KTKCVFM (4 mM) at 15 °C in the absence (a) and in the presence (b) of farnesyltransferase (45 μM). The data sets consisted of 2 × 256 free induction decays of 1024 complex points each, and the spectral width was 6000 Hz in both dimensions. The mixing time was 300 ms. The data were zero-filled along the t_1 dimension to yield matrices of 1024 × 1024 real points after Fourier transformation. New cross-peaks in (b) correspond to trNOEs developed in the peptide upon binding to the enzyme. Assignments for some cross-peaks are given in Table I. The assignments for the other cross-peaks are as follows: 2, V NH–V Hγ; 5, V Hβ–F NH; 6, V NH–V Hβ; 7, M NH–M Hβ; 8, M NH–M Hγ; 10, F NH–F Hβ; 12, F Hβ–F H2,6; 13, F NH–F Hβ; 15, F Hβ–F H2,6; 17, V NH–V Hα; 20, M NH–M Hα; 22, F NH–F Hα; 24, F Hα–F H2,6; 25, F NH–F H2,6; 26, F H2,6–M NH.

RESULTS AND DISCUSSION

Assignments of all of the proton resonances of the peptide KTKCVFM at 15 °C were obtained using NOESY and TOCSY spectra. Expansions of the amide/amide and amide/aliphatic regions of the NOESY spectrum are shown in Figure 1a. Only a few cross-peaks, all corresponding to sequential $H\alpha_i/NH_{i+1}$ interactions, are observed due to the rapid tumbling of this small molecule. Addition of farnesyltrans-

Table I: Selected Nuclear Overhauser Effects Observed in the Heptapeptide Substrate KTKCVFM in Its Free Form and in the Presence of Protein Farnesyltransferase[a]

	magnitudes of NOE cross-peaks[b]		
		peptide with enzyme at mixing time	
proton pair[c]	free peptide[d]	150 ms	300 ms
K1 Hα–T NH (19)	0.64	1.33	1.41
T Hα–K3 NH (18)	0.75	1.50	1.71
C Hα–V NH (21)	1.04	2.69	2.95
C Hβ–V NH (9)		0.65	0.46
V NH–F NH (28)		0.67	0.71
V Hα–F NH (16)	0.75	2.25	2.85
V Hγ–F NH (1)		0.71	0.87
V Hγ–F H3,5 (3)		0.33	0.52
V Hγ–F H2,6 (4)		0.36	0.69
F NH–M NH (27)		0.59	0.75
F Hα–M NH (23)		1.87	2.49
F Hβ–M NH (11)		0.30	0.44
F Hβ–M NH (14)		0.47	0.47

[a] Experimental conditions are as described in Materials and Methods. Interresidue NOEs which were present at two mixing times (150 and 300 ms) are listed. [b] The volume integrals given are for cross-peaks in the upper left quadrant, as the excitation profile of the pulse sequence used introduces asymmetry and substantially reduces the intensities of some of the corresponding cross-peaks below the diagonal (Plateau & Guéron, 1982). [c] Interproton NOEs are designated by a number in parentheses corresponding to their appearance in the spectra shown in Figure 1. [d] Mixing time 300 ms.

ferase gives rise to many new cross-peaks that correspond to trNOEs developed in the bound state of the peptide (Figure 1b). Assignment of the trNOEs to proton pairs in the peptide substrate thus enables analysis of the bound conformation. Most of the trNOEs arise from the CVFM portion of the molecule, arguing that this segment binds most strongly and is consequently most immobilized by its interaction with the enzyme. In fact, even the conformationally independent intraresidue trNOEs arising from the KTK segment of the peptide are absent. These observations strongly support a specific binding of the CVFM portion of the sequence to the enzyme. This result is consistent with the many findings that implicate primarily the C-terminal tetrapeptide sequence in determining the specificity of prenyl transferases (Clarke, 1992; Seabra et al., 1991; Chen et al., 1991).

Thirteen interresidue trNOEs (Table I) and 30 intraresidue trNOEs (data not shown) were observed. We note in particular the highly diagnostic NH_i/NH_{i+1} NOEs between the Val and the Phe and the Phe and the Met residues and the NOEs between the Val and the Phe side chains. These interactions require a reversal in the polypeptide chain direction, as in a helix or a β turn (Wüthrich, 1986). An α-helical conformation is unlikely to be present in such a short stretch of polypeptide chain and can be eliminated due to the absence of other neighboring NH_i/NH_{i+1} trNOEs. The presence of both Val NH/Phe NH and Phe NH/Met NH trNOEs argues that the turn adopted in the bound state is a type I β turn with Val and Phe in the corner positions. Indeed, computer graphics shows that the two amide/amide interactions and the Val Hγ/Phe NH, Val side-chain/Phe side-chain, and Phe Hβ/Met NH trNOEs can all be very easily accommodated in a type I β-turn conformation.

In addition to the new NOEs observed in the peptide upon addition of the farnesyltransferase (all of which are in the CVFM part of the molecule), the sequential $H\alpha_i/NH_{i+1}$ NOEs for the entire peptide are strongly enhanced upon addition of farnesyltransferase (Figure 1 and Table I). The strong $H\alpha_i/NH_{i+1}$ trNOEs cannot arise from the same conformation that yields NH_i/NH_{i+1} and $H\beta(H\gamma)_i/NH_{i+1}$ trNOEs.[2] This inconsistency is unlikely to originate from spin diffusion, as all the trNOEs listed in Table I are present at both 150- and 300-ms mixing times. Note also that (i) the $H\alpha_i/NH_{i+1}$ trNOEs are the only interresidue trNOEs that are not consistent with the proposed conformation, (ii) these trNOEs correspond to the only interresidue NOEs observed in the free peptide, and (iii) the enhancement of the $H\alpha_i/NH_{i+1}$ NOEs upon addition of farnesyltransferase occurs along the whole sequence. Possible explanations for the strong intensities of the $H\alpha_i/NH_{i+1}$ trNOEs include an increase in the viscosity of the solution upon addition of the protein and nonspecific binding of the peptide to the surface of the protein.[3] A general enhancement of the NOEs present in the extended conformations that predominate in the free peptide may well result from prebinding of the peptide (in an extended conformation) near the binding site, followed by release of the peptide before all the interactions with the active site are fully engaged and the preferred bound conformation is adopted. Such enhancements of the sequential $H\alpha_i/NH_{i+1}$ NOEs in trNOE experiments may be quite general. For example, we observed that peptides that bind to the molecular chaperone GroEL in an α-helical conformation (Landry & Gierasch, 1992) also have stronger $H\alpha_i/NH_{i+1}$ trNOEs than expected for a helical conformation, and a similar phenomenon appears to occur in the interaction of a troponin I derived peptide with troponin C.[4] At its origin, this effect may arise from the combination of a very short $H\alpha_i/NH_{i+1}$ distance in the extended state (2.2 Å) and the high likelihood of a significant population of the extended state in any unstructured peptide or in a set of nonspecifically bound peptides. Hence, quantitative interpretation of trNOEs, particularly those that correspond to NOEs present in the free ligand, in terms of the bound conformation(s) of the ligand must be done with caution.

Despite these limitations, trNOE information can be effectively used in a qualitative way, with the help of computer modeling, to develop a model for the conformation of the

[2] Due to the tetrahedral nature of the α carbons in the peptide backbone, there is no single conformation possible where the Hα, the NH, and the side chain of a residue can be oriented at the same time toward the NH of the following residue. Hence, the strong intensity of the $H\alpha_i/NH_{i+1}$ trNOEs is *per se* incompatible with the presence, at the same time, of NH_i/NH_{i+1} and $H\beta(H\gamma)_i/NH_{i+1}$trNOEs.

[3] In trNOE experiments, it is desirable to establish that those trNOEs used to deduce the bound conformation arise from specific binding. To this end, we used three different peptides as "controls", but the results, while informative, were not definitive. In one, the Cys and the Val residues were reversed in the sequence (KTKVCFM). In another, the Cys was replaced by Ser (KTKSVFM). In the third, the sequence was changed completely but retained the same amino acid composition (FKCTMKV). All of the control peptides had very low inhibitory activity, with concentrations of >100 μM required for half-maximal inhibition in our standard assay (Reiss et al., 1991). The peptide with the Val and the Cys reversed, *viz.*, KTKVCFM, developed only weak trNOEs upon addition of farnesyltransferase (data not shown), arguing that its binding to farnesyltransferase is weak. The second control, the Cys → Ser variant, KTKSVFM, developed trNOEs comparable to the original peptide in the presence of enzyme. Surprisingly, despite its lack of similarity to the original substrate peptide, the mixed sequence peptide, FKCTMKV, still gave some weak trNOEs upon addition of enzyme. These results most likely arise because of the remaining capacity of these peptides to bind, albeit weakly, and underline a limitation of the trNOE method, *viz.*, that rather weak interactions can give rise to significant trNOEs. Under some conditions, a trNOE of the same magnitude will occur for a comparable interproton distance in ligands whose affinities range from K_D's of 10^{-3} to 10^{-7} M (Clore & Gronenborn, 1982). Thus, trNOE results must be interpreted together with complementary quantitative methods of assessing binding, such as the inhibition assay.

[4] See Figure 6 of Campbell and Sykes (1991).

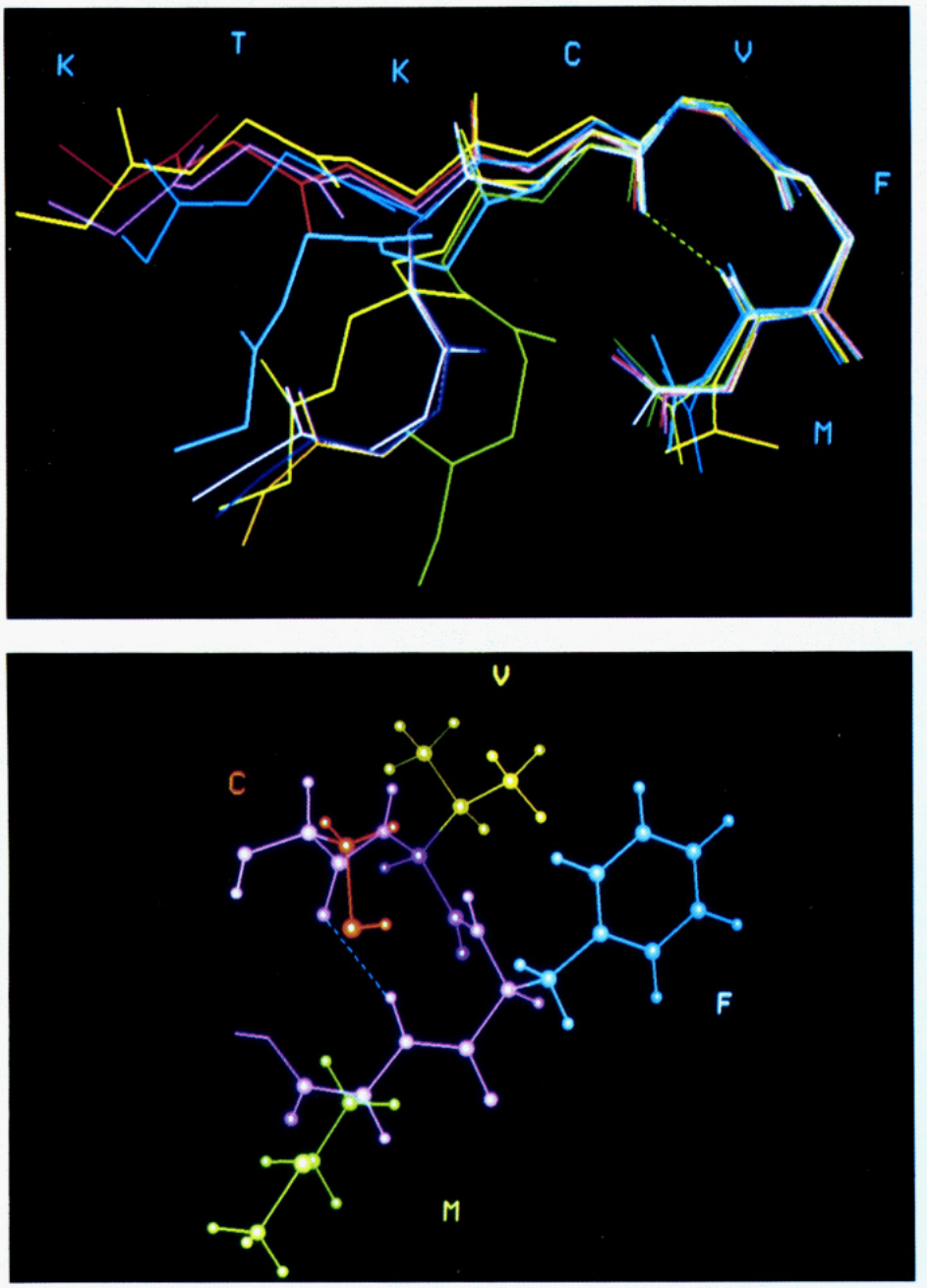

FIGURE 2: Model of the farnesyltransferase-bound form of the peptide KTKCVFM constructed by restrained molecular dynamics and energy minimization (see text). (a, top) Superposition of the backbone atoms of 10 structures taken at 2-ps intervals from a 20-ps restrained molecular dynamics trajectory. All of the structures displayed were energy-minimized using the same restraints. The superposition was obtained by minimizing the root mean square deviation between the backbone atoms of residues CVFM. The dashed line indicates a hydrogen bond between the Met carbonyl oxygen and the Cys NH, which was observed in all of the minimized structures and is characteristic of β turns. The superposition is intended to illustrate the relative mobility of the N-terminal residues, KTK, with respect to the CVFM part of the molecule and does not represent a systematic search of all structures compatitble with the trNOE data. (b, bottom) The turn region: Only the backbone atoms and the NH protons of the residues forming the turn (CVFM) are shown. The backbone atoms are in magenta, and the side chains are color-coded as follows: C, red; V, yellow; F, blue; and M, green. The structure displayed was generated from the last structure obtained in the restrained molecular dynamics simulation, by interactively manipulating the V and F side chains to fit the observed interresidue NOEs and subsequently minimizing the energy.

bound ligand. To develop an approximate picture of the conformation of KTKCVFM when bound to farnesyltransferase, we started with a completely extended conformation of the peptide and then manipulated this conformation interactively to impose a type I β turn around Val–Phe and force the *pro-S* Cys Hβ proton to be in proximity (2.5 Å) to the Val NH. After energy minimization of the resulting structure, we calculated a 20-ps molecular dynamics trajectory with restraints to keep the Val NH/Phe NH, Phe NH/Met NH, and *pro-S* Cys Hβ/Val NH distances between 2.4 and 2.9 Å.[5] Figure 2a displays the backbone conformation of energy-minimized structures visited along the trajectory. This result by no means constitutes a systematic search and includes only those interresidue trNOEs that do not suffer complications in interpretation (*e.g.*, from ambiguities such as lack of stereospecific assignment or interaction with methyl or

[5] Restraining the *pro-R* Cys Hβ/Val NH distance or using only an upper limit (2.9 Å) in the distance restraint yields a very similar backbone conformation.

aromatic protons). Nonetheless, note that the three restraints introduced are sufficient to keep the CVFM part of the molecule in a conformation very similar to the starting structure, while the KTK portion is conformationally flexible due to the lack of restraints. Figure 2b shows a model structure in the turn region, which was obtained from the last structure in the simulation by manipulating the Val and Phe side chains to satisfy qualitatively the observed interresidue trNOEs, followed by energy minimization. The relative intensities of all interresidue trNOEs (Table I), with the exception of the $H\alpha_i/NH_{i+1}$ interactions as discussed above, are all consistent with the interproton distances in the modeled structures. Thus, a semiquantitative analysis of the observed trNOEs supports our conclusion that the CVFM sequence adopts a type I β turn with the V and the F residues in the corner positions.

Several recent studies of the recognition of short peptide sequences by proteins have implicated turn conformations (Marshall, 1992). Turn conformations enable maximal accessibility of side chains in a given sequence (Rose et al., 1985). In the case of farnesyltransferase, the disposition of the CAAX side chains in a β turn provides access to reactive groups on the enzyme for the cysteine modification as well as to residues on the enzyme involved in recognition. Knowledge that the substrate presents its binding region in a turn bodes well for inhibitor design, as there is a real possibility of introducing conformational constraints in a cyclic peptide or in a peptidomimetic inhibitor (Rizo & Gierasch, 1992).

During the course of this study, an independent effort to develop a peptidomimetic inhibitor of farnesyltransferase used a benzodiazepine to replace the central two residues of CVFM. The resulting molecule inhibits Ras farnesylation both *in vitro* and in animal cells (James et al., 1993). The design of this inhibitor was based on the assumption that the CAAX sequence binds as a reverse turn. Our data have now provided direct evidence for this binding mode and offer a clear rationale for the effectiveness of the benzodiazepine inhibitor.

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