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Conformation of a T cell stimulating peptide in aqueous solution

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Using two-dimensional NMR spectroscopy and circular dichroism spectroscopy it is demonstrated that a T cell stimulating peptide corresponding to residues 132–153 of sperm whale myoglobin populates helical conformations in aqueous solution. This finding is in accordance with proposals that immunodominant sites in T cell stimulating peptides have a high conformational propensity. The observation of secondary structure in aqueous solutions of this and other immunogenic peptides has important implications for initiation of protein folding.

CD; Peptide; Helix, α-; Myoglobin; NMR, 2D; Protein folding; (T cell)

1. INTRODUCTION

The molecular basis of recognition of protein antigens by T cells is presently the subject of intense research activity. The weight of evidence indicates that protein antigens are not recognised in their native three-dimensional conformations but are processed into short peptides [1] which bind directly to an MHC protein for presentation to the T cell receptor [2,3]. The X-ray structure of the human class I MHC protein, HLA-A2, reveals a probable peptide binding site formed by 2 antiparallel α-helices and a region of β-sheet [4,5]. It has been proposed [6] that structurally similar binding sites are present also in class II MHC proteins. The number of different MHC proteins in a given individual is limited yet each must recognise and bind with an extremely wide range of processed peptides derived from foreign antigens. This raises important questions about the structural requirements for T cell activation by peptides. However, to date, there is no experimental evidence concerning the conformation(s) adopted by T cell epitopes in the proposed binding site or in solution, both of which are of course pertinent to the free energy of the binding process. Hence, we have used nuclear magnetic resonance (NMR) spectroscopy to investigate the conformational preferences of a peptide containing the I–E^d immunodominant region of sperm whale myoglobin (residues 136–145) [7]. We show that this peptide adopts significantly populated helical conformations in aqueous solution.

^{1}H-NMR spectroscopy has emerged as a powerful tool for examining the conformational preferences of linear peptides in solution. Using NMR techniques we have been able to show that several short immunogenic peptides, each capable of inducing antibodies that bind with high affinity to the cognate sequence in the folded protein, adopt highly preferred conformations in aqueous solution [8–12]. In the present work we have extended our analysis to a peptide containing an immunodominant helper T cell epitope. This peptide, Mbf1, corresponds to residues 132–153 of myoglobin, and constitutes 18 residues of the H-helix and the 4 C-terminal residues of the native protein.

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Abbreviations: NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; COSY, scalar correlated spectroscopy; TOCSY, total correlated spectroscopy; CD, circular dichroism; MHC, major histocompatibility complex

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2. MATERIALS AND METHODS

Mbf1 was prepared as described previously [13] and was purified using reverse-phase high pressure liquid chromatography. NMR samples in the concentration range 10 μM to 10 mM were prepared in 95% H_2O/5% D_2O or 100% D_2O, and the pH adjusted to 4.0. CD samples ranged from 2 μM to 2 mM. Assignment of the ^{1}H-NMR spectrum was made using a standard strategy and will be described in detail elsewhere. Two-dimensional double quantum filtered COSY and TOCSY experiments were employed to assign resonances within spin systems. These spin systems were then connected in a sequence specific manner using two-dimensional NOESY. Mixing times for the NOESY experiments were varied from 100 to 500 ms to identify both shorter and longer range interproton distances, and to assess the extent of spin diffusion. As observed previously for small linear peptides [10,11], spin diffusion was not significant even at the longer mixing times employed.

Small linear peptides do not adopt unique conformations in aqueous solution but sample a number of conformational states. The problem of conformational analysis for linear peptides has been discussed in [12]. The most important NMR parameter for determination of peptide conformation is the NOE, the rate of build-up of which is dependent on the inverse 6th power of the relevant interproton distance. Extended conformations are characterised [13] by NOEs between the C$^\alpha$H proton of residue i and the backbone amide proton of residue $i+1$, termed $d_{\alpha N}(i,i+1)$ NOE connectivities. Helical conformations are characterised by $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$ NOE connectivties [13]. Whilst NOEs are highly sensitive indicators of folded structures within a conformational ensemble, it is extremely difficult to determine the populations of these conformations from the relative NOE build-up rates. The inverse 6th power weighting of the NOE means, for example, that the NOEs of extended conformations, with a $d_{\alpha N}(i,i+1)$ distance of 2.2 Å [13] (cf. a $d_{NN}(i,i+1)$ distance of 2.8 Å in an α-helix [13]), will be detected even if the population of extended conformations is relatively small. In contrast, the threshold population for the observation of medium-range $(i,i+3)$ NOEs characteristic of helical conformations is relatively high (assuming similar correlation times for the various populated conformations). In the present work, spectra are used in a purely qualitative sense to identify conformations whose populations in aqueous solution exceed the threshold value for the observation of diagnostic NOEs.

3. RESULTS AND DISCUSSION

Regions of a NOESY spectrum of Mbf1 containing the $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+1)$ NOEs and the intraresidue $d_{N\alpha}(i,i)$ NOEs are shown in fig.1. One immediately striking feature of this spectrum is that the ratio of the itensity of $d_{NN}(i,i+1)$ to $d_{\alpha N}(i,i+1)$ NOEs is uncommonly large in this peptide compared with many peptides corresponding to secondary structural elements (including helices) from other proteins [10,11]. The NOEs observed

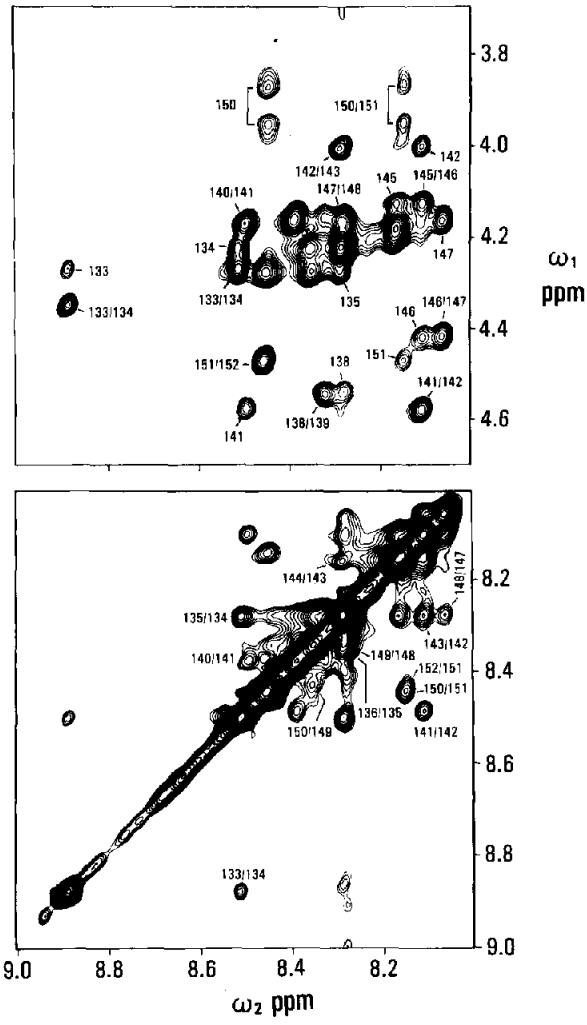

Fig.1: Two regions of a NOESY spectrum of Mbf1 showing above, $d_{\alpha N}$ NOE connectivities, and below, d_{NN} NOE connectivities plotted at the same contour levels. The crosspeaks are identified by the amino acid residue number measured in the ω_1 dimension followed by that measurement in the ω_2 dimension. For example, in the upper region the crosspeak denoted 133/134 is the $d_{\alpha N}(i,i+1)$ NOE between the C$^\alpha$H of K133 and the backbone amide proton of A134, and the crosspeak denoted 133 is the $d_{\alpha N}(i,i)$ NOE within residue K133. In the lower region, the crosspeak denoted 133/134 is the $d_{NN}(i,i+1)$ NOE between the backbone amide protons of K133 and A134. The spectrum shown was acquired using an 11 mM solution of Mbf1 in 90% H_2O/10% D_2O at pH 4.0 and 278 K. The mixing time (τ_m) was 200 ms. Similar NOESY data sets were obtained at concentrations ranging from 1 to 6 mM, and no changes in the relative NOE intensities were observed.

for Mbf1 are summarized in fig.2 and are characteristic of both helical and extended (β) conformations. A significant population of helical

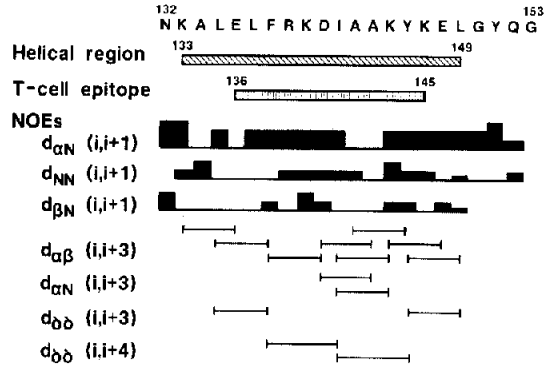

Fig.2. Amino acid sequence of Mbf1 (residues 132–153 of sperm whale myoglobin) showing the region which adopts helical conformations and the location of the T cell epitope. The observed NOE connectivities are also shown. Sequential $(i,i+1)$ NOEs are represented by filled blocks under residues i. The height of the block is an approximate measure of NOE intensity after a mixing time of 200 ms. In cases where resonance overlap prevents the unambiguous observation of an NOE a line is present under residue i. Where no NOE is observed the space is left blank. Medium range $(i,i+3)$ and $(i,i+4)$ NOEs are represented by bars connecting the appropriate residues.

conformations is indicated by the sequence of $d_{NN}(i,i+1)$ NOE connectivities between consecutive residues (see fig.1) and by the medium range $d_{\alpha\beta}(i,i+3)$ and $d_{\alpha N}(i,i+3)$ NOE connectivities [13]. The region of a NOESY spectrum of Mbf1 containing $d_{\alpha\beta}(i,i+3)$ NOEs is shown in fig.3. The $d_{\alpha\beta}(i,i+3)$ NOE between D141 $C^{\alpha}H$ and A144 $C^{\beta}H_3$ is particularly intense, building up at approx. 1/5th the rate of the intraresidue $d_{\alpha\beta}(i,i)$ NOE of D141. Indeed, in a helical conformation, the maximum $d_{\alpha\beta}(i,i+3)$ distance is shortest (2.5–3.1 Å) [13] when the $C^{\beta}H_3$ group of alanine is involved.

The observed $d_{NN}(i,i+1)$ and the medium range $(i,i+3)$ NOE connectivities (fig.2) indicate that the peptide populates helical conformations between residues 133 and 149. This conclusion is supported by the distribution of $d_{\beta N}$ $(i,i+1)$ NOEs (fig.2), which are a consequence of (but not exclusive to) helical conformations [13]. In addition of the NOE connectivities characteristic of helical conformations, $d_{\alpha N}(i,i+1)$ NOE connectivities characteristic of extended chain conformations [13] are observed throughout the peptide. These $d_{\alpha N}(i,i+1)$ NOEs build up at a rate on average only approx. twice faster than the $d_{NN}(i,i+1)$ NOEs. NOEs characteristic of extended conformations normally

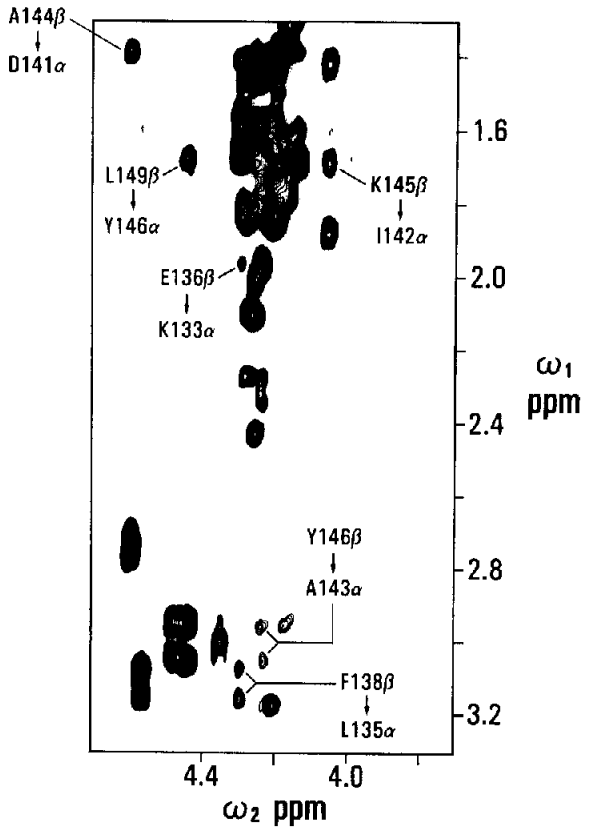

Fig.3. The $d_{\alpha\beta}$ NOE region of a NOESY spectrum run under identical conditions to that shown in fig.2., but using a 1.5 mM sample and a mixing time of 400 ms. Indicated are the resolvable $d_{\alpha\beta}(i,i+3)$ connectivities, i.e. those characteristic of helical conformations. The NOE K145 $C^{\alpha}H$/E148 $C^{\beta}H$ is only just visible at the contour level plotted, and the NOE F138 $C^{\alpha}H$/D141 $C^{\beta}H$ is only resolvable from the NOE D141 $C^{\alpha}H$/D141 $C^{\beta}H$ using high resolution window functions in the F2 dimension, and at higher temperatures. The presence of the $d_{\alpha\beta}(i,i+3)$ NOEs at concentrations ranging from 0.5 to 11 mM was confirmed using truncated-driven NOE difference spectra. Pseudo-2D truncated-driven NOESY experiments [29] were employed where resonance overlap was a problem.

dominate the NOESY spectra of unfolded ('random coil') peptides in aqueous solution [10–12].

The NMR experiments by themselves cannot distinguish between single isolated helical turns and helices involving several turns. However, according to the helix-coil transition theory [14,15], it is unlikely that isolated helical turns would be sufficiently stable to account for the observed NOE connectivities. Evidence for a small population of helix was obtained from circular dichroism (CD) measurements. The CD spectra of Mbf1 in

aqueous solution show negative ellipticity at 222 nm ($\theta_m = -2800$ deg·cm^2·dmol^{-1}) but no pronounced minimum, in accord with previous studies [16,17]. However on addition of 9 M guanidinium hydrochloride θ_m increases to $+2400$ deg·cm^2·dmol^{-1}, suggesting the presence of a small but significant population of helix in water solutions of Mbf1. On substitution of Tyr 146 by lysine and acetylation of the N-terminus the CD spectrum shows a characteristic minimum at 222 nm ($\theta_m = -10\,700$ deg·cm^2·dmol^{-1}) indicative of helix, although no discernible changes in NOE build-up rates were observed relative to the native Mbf1 peptide. We conclude from the NMR and CD results that both Mbf1 and the Lys 146 analogue adopt helical conformations (between residues 133 and 149) which are in rapid exchange with unfolded states. We note that early immunological studies of Mbf1 also suggested a small population of helical conformations [18]. Mbf1 is one of only a very few peptides which have been shown to adopt helical conformations in aqueous solution [11,12,15].

The CD and NMR spectra of Mbf1 are concentration independent over a wide range (2 μM to 1.5 mM for CD, 10 μM to 1.5 mM for NMR). The peptide adopts helical conformations at these concentrations and gel filtration experiments using Biogel P6 columns show that it is monomeric. This is confirmed by measurement of the linewidths of backbone NH, $C^{\alpha}H$ and aromatic ring proton resonances which, together with correlation times calculated from NOE build-up rates, are comparable to those of monomeric melittin (M_r 2840, compared to M_r 2553 for the monomer of Mbf1) and are very much smaller than those of tetrameric melittin or bovine pancreatic trypsin inhibitor (M_r 6512). At concentrations higher than 1.5 mM, some changes in chemical shifts and small increases in resonance linewidth suggest that some intermolecular association is occurring. Of great importance, however, the NOEs characteristic of the helical conformations are not measurably changed in relative intensity over the concentration range 0.5–11 mM. Thus intermolecular association is not the driving force for helix formation.

In addition to the NOEs reported above, a number of medium range sidechain–sidechain NOEs are observed within the helical region of the peptide (see fig.2). These include a family of NOEs that connects the δ-protons of hydrophobic residues with both $(i,i+3)$ and $(i,i+4)$ relationships. The presence of these NOEs emphasises the close packing of hydrophobic sidechains created by helix formation.

4. CONCLUSION

On the basis of statistical analysis it has been suggested that immunodominant sites in T cell stimulating peptides have a high conformational propensity [19] and several workers have proposed that the ability to form secondary structure is important for antigenicity [20–26]. In particular, Berzofsky and co-workers [27] have shown that 18 out of 22 known immunodominant helper T cell epitopes correspond to sequences that are predicted to form stable amphipathic helices. This paper presents direct evidence that one of these epitopes, comprising residues 136–146 of sperm whale myoglobin, has such a high propensity to adopt helical conformations that it does so in aqueous solution. While these observations lend support for the proposed role of amphipathic helix formation in T cell immunogenicity, it must be emphasized that not all known T cell epitopes are predicted to form amphipathic helices and other structural characteristics may in some cases be important.

We have proposed elsewhere [8,12,28] that there is a relationship between B cell immunogenicity and peptide folding and have suggested [8,10,11] an immunological approach to identification of protein folding initiation sites. The present results are strikingly analogous and suggest that this approach may extend also to T cell immunogens. Further experiments are being carried out to investigate the factors which stabilise helical structures in the Mbf1 peptide. NMR experiments on immunogenic peptides are providing new insights into the role of the amino acid sequence in stabilization of secondary structures in peptide fragments of proteins. Such structures may play a fundamental role in initiation of protein folding and direction of folding pathways [12].

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