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Anomalous reversed-phase high-performance liquid chromatographic behavior of synthetic peptides related to antigenic helper T cell sites

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ABSTRACT

Sets of overlapping synthetic peptides for three well characterized proteins (sperm whale myoglobin, hen egg lysozyme, and the circumsporozoite protein from *Plasmodium falciparum*) were prepared and examined by reversed-phase high-performance liquid chromatography (RP-HPLC). Using retention coefficients to predict the retention time of each peptide, several peptides in each protein set were found that exhibited anomalous behavior (*i.e.* eluted significantly later than predicted). Previous work with model peptides has shown that this anomalous behavior can be attributed to specific amphipathic arrangements induced by the lipid stationary phase during the RP-HPLC process. In the current study it was found that although not all of the peptides containing an antigenic T cell site displayed anomalously late behavior, all of the peptides which eluted anomalously late during RP-HPLC included the regions of these proteins known from earlier studies to be antigenic T cell sites.

INTRODUCTION

T cell immune response involves a multi-step process that begins with the internalization of a foreign protein antigen by antigen presenting cells (APC). The protein antigen is then unfolded and/or processed into smaller peptide fragments by a still incompletely understood mechanism. These peptide fragments are then presented in association with class I or class II major histocompatilitity complex (MHC) membrane protein on the surface of the APC. The specific T cell receptors that recognize the peptide antigen-MHC protein bimolecular complex initiate T cell activation and proliferation. It has been proposed that the processed antigen adopts, maintains, and/or is induced into a specific conformation which is stabilized by hydrophobic forces upon its interaction with MHC [1,2]. In a recent review [3], however, evidence was presented for and against the fact that T cell determinants adopt specific secondary structures. Also, a number of prediction programs based on secondary structural motifs have been developed to identify potential antigenic T cell sites [1,4–7]. Nevertheless, the fact that T cell recognition requires that a protein antigen be processed, and ultimately presented as defined peptide fragments, permits the use of synthetic peptides for the study of the structural requirements of antigenic T cell sites.

In aqueous solution, peptides exist in a vast number of conformational arrays which are ultimately dependent upon a given peptide's amino acid sequence. However, in lipid or other hydrophobic environments, peptides display highly ordered and/or stabilized conformations [8]. We and others have found that reversed-phase high performance liquid chromatography (RP-HPLC) is a simple and useful

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tool for the study of induced secondary structure of peptides [9–12]. As a simple model system, the aqueous mobile phase and the octadecyl (C_{18}) of the stationary phase can be considered to mimic the anisotropic aqueous–lipid environment which exists when peptides interact with membranes. We reasoned that if a correlation could be found between induced secondary structure of peptides (as determined by RP-HPLC retention times) and antigenic T cell sites (given their postulated tendency to adopt α-helical amphipathic structures [1]) then RP-HPLC could be used to determine the antigenic T cell sites of proteins.

Retention coefficients have been widely used for the prediction of the retention times of peptides during RP-HPLC [13,14]. These values, which are based solely on the amino acid composition (irrespective of sequence) of a peptide, involve the assignment of specific, empirically determined hydrophobicity values for each amino acid. We have found, however, that a significant number of peptides exhibit anomalous RP-HPLC behavior relative to their predicted retention times [15,16]. We reasoned that differences between the theoretically calculated and experimentally determined retention times must result from specific conformational effects induced during the RP-HPLC process. For instance, in studies involving model peptides expected to have amphipathic conformations when induced into an α-helix, decreases in RP-HPLC retention times correlated well with the expected perturbations in amphipathicity caused by single residue substitutions [12,17–19].

In the current study we prepared complete sets of overlapping peptides for three proteins for which helper T cell determinants have been identified, namely sperm whale myoglobin (SWM), hen egg lysozyme (HEL), and the circumsporozoite protein (CSP) of *Plasmodium falciparum*. The RP-HPLC behavior of the peptides in each series (*i.e.*, the differences found between predicted and experimentally determined retention times) was then correlated with the MHC class II antigenic helper T cell sites for each of these three proteins.

EXPERIMENTAL

Peptide synthesis

Peptide resins were synthesized using the method of simultaneous multiple peptide synthesis [20] using Boc chemistry. Twenty-four peptide resins were cleaved simultaneously by liquid hydrogen fluoride using a multiple vessel cleavage apparatus [21]. Peptide purity was determined by analytical RP-HPLC (pH 2.1). Purities of the crude peptides ranged from 70 to 85%. No further purification or analyses were carried out. Numbering for each set of overlapping synthetic peptides was begun at the N-terminus of each of the three proteins. The peptides for the SWM and HEL were 15 residues in length and overlapped by 5 residues (*i.e.*, 1–15, 6–20, 11–25, etc.), resulting in a total of 29 peptides for SWM and 24 peptides for HEL. In the case of CSP 22, 20-residue peptides were prepared which overlapped by 10 residues(*i.e.*, 1–20, 11–30, etc.). The central repeat region of the protein (NANP 121–292) was not included.

Reversed-phase high-performance liquid chromatography

The RP-HPLC system utilized consisted of two Altex Model 110A pumps, a Beckman Model 421 microprocessor (Beckman Instruments, Anaheim, CA, USA), a Hitachi Model 100-20 variable-wavelength spectrophotometer (Baxter Scientific Products, Los Angeles, CA, USA), a Shimadzu CR3A integrator (Cole Scientific, Calabasas, CA, USA) and a Bio-Rad Model AS-48 autosampler (Bio-Rad Labs., Richmond, CA, USA). Samples (20 μl, 0.2 mg/ml) were analyzed on Vydac 218TP54 C_{18} columns, 250 mm × 4.6 mm I.D., 5 μm particle size (Alltech Assoc., Los Altos, CA, USA), and a solvent system consisting of buffer A (0.01 *M* $(NH_4)_2HPO_4$, pH 7.0) and buffer B (acetonitrile) was used. A 1% gradient consisting of 5% B to 50% B in 45 min was used throughout. Elution was measured at 215 nm. The experimentally determined retention times of each peptide were normalized by the inclusion of the same peptide reference standard in each run to eliminate deviations in the results due to variations in column performance and injection times.

Retention time prediction

A modification based on Meek's procedure [22] for the calculation of the individual retention coefficients for each amino acid was used. The differences in predicted and experimentally determined reten-

tion times were averaged and a standard deviation was calculated for each set of synthetic peptides associated with the three proteins. Peptides found to have differences in retention times that exceeded ±1 S.D. standard deviation from the average were considered to exhibit anomalous elution behavior during the RP-HPLC process.

RESULTS

Sperm whale myoglobin

The RP-HPLC elution behavior for the 29 overlapping peptides of SWM are shown in Table I. The average difference between the experimentally determined and predicted retention times (Δ retention time) of the peptides of the SWM series were found to be −0.8 min with an S.D. of 3.3 min (Fig. 1). RP-HPLC elution behavior within ±1 S.D. of the average was found for 22 of the 29 peptides. Four peptides eluted significantly later than predicted in the SWM series, namely, peptide 4 (SWM 16–30), peptide 14 (SWM 66–80), peptide 22 (SWM 106–120), and peptide 26 (SWM 126–140). The majority of the residues of these 4 peptides correspond to regions of the SWM sequence that have been reported to be antigenic helper T cell sites for MHC class II (Table I, ref. 23).

Hen egg lysozyme

The RP-HPLC elution behavior of the 24 overlapping peptides of HEL are shown in Table II. The differences between the experimentally determined

TABLE I

RETENTION TIMES FOR OVERLAPPING PEPTIDES OF SWM

No.	Peptide residue	Sequence	RP-HPLC retention time (min)			Known T cell site[a]
			Experimental	Predicted	Experimental − predicted	
1	1–15	VLSEGEWQLVLHVWA-NH_2	28.5	32.6	−4.1	
2	6–20	EWQLVLHVWAKVEAD-NH_2	28.9	28.5	0.4	
3	11–25	LHVWAKVEADVAGHG-NH_2	23.7	23.4	0.2	
4	16–30	KVEADVAGHGQDILI-NH_2	23.7	20.3	3.4	15–22
5	21–35	VAGHGQDILIRLFKS-NH_2	31.0	29.5	1.5	
6	26–40	QDILIRLFKSHPETL-NH_2	29.8	29.6	0.2	
7	31–45	RLFKSHPETLEKFDR-NH_2	20.3	25.0	−4.7	
8	36–50	HPETLEKFDRFKHLK-NH_2	21.4	25.1	−3.8	
9	41–55	EKFDRFKHLKTEAEM-NH_2	21.8	22.2	−0.4	
10	46–60	FKHLKTEAEMKASED-NH_2	17.6	17.7	−0.1	
11	51–65	TEAEMKASEDLKKHG-NH_2	10.2	13.6	−3.3	
12	56–70	KASEDLKKHGVTVLT-NH_2	18.2	20.9	−2.7	
13	61–75	LKKHGVTVLTALGAI-NH_2	30.2	29.8	0.5	
14	66–80	VTVLTALGAILKKKG-NH_2	38.7	29.8	8.9	70–78
15	71–85	ALGAILKKKGHHEAE-NH_2	20.2	20.7	−0.5	
16	76–90	LKKKGHHEAELKPLA-NH_2	17.2	21.4	−4.3	
17	81–95	HHEAELKPLAQSHAT-NH_2	15.3	17.9	−2.7	
18	86–100	LKPLAQSHATKHKIP-NH_2	15.5	24.4	−8.9	
19	91–105	QSHATKHKIPIKYLE-NH_2	21.4	23.4	−2.0	
20	96–110	KHKIPIKYLEFISEA-NH_2	28.4	28.3	0.1	
21	101–115	IKYLEFISEAIIHVL-NH_2	35.1	34.6	0.6	
22	106–120	FISEAIIHVLHSRHP-NH_2	35.1	30.2	4.9	106–118
23	111–125	IIHVLHSRHPGDFGA-NH_2	26.3	27.4	−1.1	
24	116–130	HSRHPGDFGADAQGA-NH_2	12.9	15.2	−2.3	
25	121–135	GDFGADAQGAMNKAL-NH_2	16.2	19.3	−3.0	
26	126–140	DAQGAMNKALELFRK-NH_2	28.1	23.1	5.0	132–153
27	131–145	MNKALELFRKDIAAK-NH_2	25.6	26.4	−0.9	
28	136–150	ELFRKDIAAKYKELG-NH_2	21.1	24.6	−3.5	
29	141–153	DIAAKYKELGYQG-NH_2	18.2	19.0	−0.8	

[a] Reported helper T cell sites as reviewed by Millich [23].

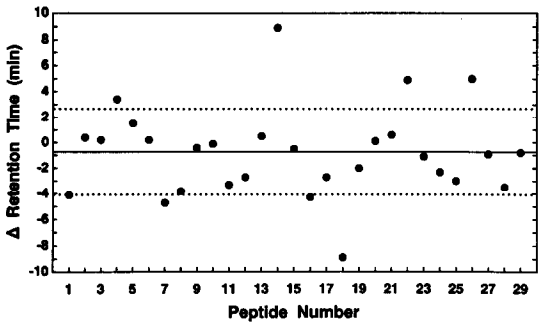

Fig. 1. RP-HPLC elution behavior for overlapping peptides of SWM. Each solid circle represents differences between the experimental retention time and the predicted retention time for each peptide. The solid line represents the average of the differences in the retention times for this set of peptides. The dotted lines represent ±1 S.D. from the average.

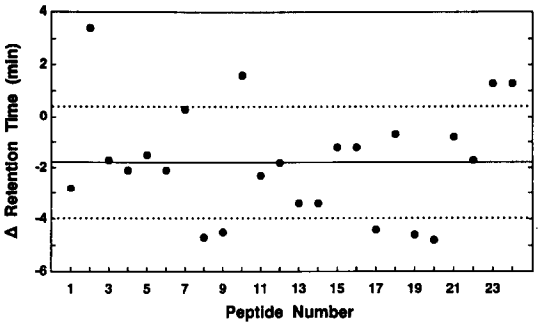

Fig. 2. RP-HPLC elution behavior for overlapping peptides of HEL. Each solid circle represents differences between the experimental retention time and the predicted retention time for each peptide. The solid line represents the average of the differences in the retention times for this set of peptides. The dotted lines represent ±1 S.D. from the average.

and predicted retention times for each peptide of the HEL series were found to average −1.8 min with an S.D. of 2.2 min (Fig. 2). RP-HPLC elution behavior for 15 of the 24 peptides fell within ±1 S.D. (2.2 min) of the average (−1.8 min). Four peptides eluted significantly later than predicted, name-

TABLE II

RETENTION TIMES FOR OVERLAPPING PEPTIDES OF HEL

No.	Peptide residue	Sequence	RP-HPLC retention time (min)			Known T cell site[a]
			Experimental	Predicted	Experimental − predicted	
1	1–15	KVFGRCELAAAMKRH-NH_2	23.1	26.0	−2.8	
2	6–20	CELAAAMKRHGLDNY-NH_2	25.2	21.8	3.4	1–18
3	11–25	AMKRHGLDNYRGYSL-NH_2	22.1	23.9	−1.7	
4	16–30	GLDNYRGYSLGNWVC-NH_2	25.0	27.0	−2.1	
5	21–35	RGYSLGNWVCAAKFE-NH_2	26.5	28.0	−1.5	
6	26–40	GNWVCAAKFESNFNT-NH_2	24.5	26.7	−2.1	
7	31–45	AAKFESNFNTQATNR-NH_2	20.7	20.4	0.3	
8	36–50	SNFNTQATNRNTDGS-NH_2	10.8	15.6	−4.7	
9	41–55	QATNRNTDGSTDYGI-NH_2	10.7	15.2	−4.5	
10	46–60	NTDGSTDYGILQINS-NH_2	20.6	18.9	1.6	46–61
11	51–65	TDYGILQINSRWWCN-NH_2	28.7	31.0	−2.3	
12	56–70	LQINSRWWCNDGRTP-NH_2	26.0	27.8	−1.8	
13	61–75	RWWCNDGRTPGSRNL-NH_2	22.5	25.9	−3.4	
14	66–80	DGRTPGSRNLCNIPC-NH_2	17.0	20.5	−3.4	
15	71–85	GSRNLCNIPCSALLS-NH_2	25.8	27.0	−1.2	
16	76–90	CNIPCSALLSSDITA-NH_2	24.2	25.4	−1.2	
17	81–95	SALLSSDITASVNCA-NH_2	18.6	23.0	−4.4	
18	86–100	SDITASVNCAKKIVS-NH_2	21.1	21.9	−0.7	
19	91–105	SVNCAKKIVSDGDGM-NH_2	14.5	19.1	−4.6	
20	96–110	KKIVSDGDGMNAWVA-NH_2	17.9	22.7	−4.8	
21	101–115	DGDGMNAWVAWRNRC-NH_2	23.3	24.2	−0.8	
22	106–120	NAWVAWRNRCKGTDV-NH_2	24.2	26.0	−1.7	
23	111–125	WRNRCKGTDVQAWIR-NH_2	28.9	27.6	1.3	112–129
24	116–129	KGTDVQAWIRGCRL-NH_2	27.2	25.9	1.3	112–129

[a] Reported helper T cell sites as reviewed by Millich [23].

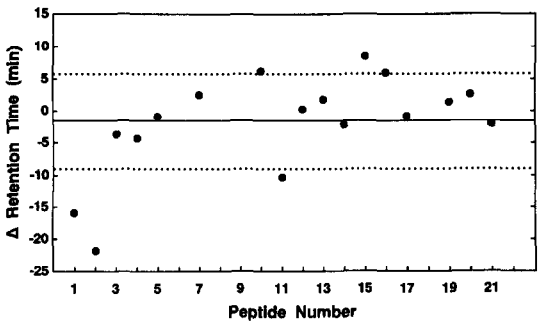

Fig. 3. RP-HPLC elution behavior for overlapping peptides of CSP. Each solid circle represents differences between the experimental retention time and the predicted retention time for each peptide. The solid line represents the average of the differences in the retention times for this set of peptides. The dotted lines represent ±1 S.D. from the average.

ly peptide 2 (HEL 6–20), peptide 10 (HEL 46–60), peptide 23 (HEL 111–125), and peptide 24 (HEL 116–129). The majority of the residues in each of these peptides are found in known HEL antigenic helper T cell sites for MHC class II (Table II, ref. 23).

Circumsporozoite protein

The RP-HPLC elution behavior of the 22 overlapping peptides of CSP are shown in Table III. The differences between the experimentally determined and predicted retention times for each peptide of the CSP series were found to average −1.8 min with a standard deviation of 7.4 min [Fig. 3]. RP-HPLC elution behavior was found for 11 of the 22 peptides fell within ±1 S.D. (7.4 min) of the average (−1.8 min). Peptides 10 (CSP 91–110), 15 (321–340), and 16 (331–350) eluted significantly later than predicted. The regions of CSP represented by peptides 15 and 16 correspond to the antigenic helper T cell sites found for both mice (IA^k) and humans (Table III, refs. 24 and 25). CSP 91–110 borders a region that is a known antigenic T cell site in hu-

TABLE III

RETENTION TIMES FOR OVERLAPPING PEPTIDES OF CSP

No.	Peptide residue	Sequence	RP-HPLC retention time (min)			Known T cell site[a]
			Experimental	Predicted	Experimental − predicted	
1	1–20	MMRKLAILSVSSFLFVEALF-NH_2	33.2	29.1	−15.9	
2	11–30	SSFLFVEALFQEYQCYGSSS-NH_2	13.1	34.9	−21.8	
3	21–40	QEYQCYGSSSNTRVLNELNY-NH_2	19.9	23.6	−3.7	
4	31–50	NTRVLNELNYDNAGTNLYNE-NH_2	18.3	22.5	−4.3	
5	41–60	DNAGTNLYNELEMNYYGKQE-NH_2	18.8	19.7	−0.9	
6	51–70	LEMNYYGKQENWYSLKKNSR-NH_2	N.D.[b]	26.2		
7	61–80	NWYSLKKNSRSLGENDDGNN-NH_2	21.1	18.6	2.5	
8	71–90	SLGENDDGNNNNGDNGRFGK-NH_2	N.D.	4.5		
9	81–100	NNGDNGREGKDEDKRDGNNE-NH_2	N.D.	0.6		
10	91–110	DEDKRDGNNEDNEKLRKPKH-NH_2	11.9	5.7	6.2	103–122
11	101–120	DNEKLRKPKHKKLKQPGDGN-NH_2	5.6	15.8	−10.3	
12	291–310	NQGNGQGHNMPMDPNRNVDE-NH_2	12.6	12.3	0.3	
13	301–320	PNDPNRNVDENANANNAVKN-NH_2	11.6	9.8	1.8	
14	311–330	NANANNAVKNNNNEEPSDKH-NH_2	5.9	8.1	−2.2	
15	321–340	NNNEEPSDKHIEQYLKKIKN-NH_2	24.4	15.9	8.5	326–343
16	331–350	IEQYLKKIKNSISTEWSPCS-NH_2	34.2	28.2	6.0	326–343
17	341–360	SISTEWSPCSVTCGNGIQVR-NH_2	25.3	26.1	−0.8	
18	351–370	VTCGNGIQVRIKPGSANKPK-NH_2	N.D.	24.1		
19	361–380	IKPGSANKPKDELDYENDIE-NH_2	14.8	13.4	1.4	
20	371–390	DELDYENDIEKKICKMEKCS-NH_2	18.4	15.8	2.6	
21	381–400	KKICKMEKCSSVFNVVNSSI-NH_2	27.7	29.8	−2.1	
22	391–412	SVFNVVNSSIGLIMVLSFLFLN-NH_2	N.D.	49.2		

[a] Reported helper T cell sites as reviewed by Millich [23].
[b] N.D. = Not determined.

mans [26]. The experimental retention times could not be determined in this RP-HPLC system utilized for peptides 6, 8, 9, 18, and 22 due to insolubility or extreme hydrophilicity.

DISCUSSION

In earlier studies, RP-HPLC was found to be useful in the determination of induced secondary structures of peptides [10,12,17–19]. In the current study, this method was extended to examine the correlation of induced peptide structure as evidenced by anomalous RP-HPLC retention time behavior with the known antigenic T cell sites of three proteins. SWM, HEL, and CSP were chosen for this study because their T cell sites have been extensively characterized for both mice and humans (reviewed in ref. 23). The predicted retention time of each peptide used in this study was calculated using retention coefficients [13,14,22]. It was hoped that this approach would permit RP-HPLC to be used as a functional model system for the interaction of peptides with class II MHC as well as for the prediction of T cell sites in proteins.

Peptides with an intrinsic potential for the adoption of amphipathic α-helical conformations have been shown to have increased retention times relative to their elution times calculated using retention coefficients [12,17–19]. This behavior can be explained if one assumes that a specific amphipathic secondary structure for these peptides is induced during hydrophobic interaction of their side chains with the C_{18} of the stationary phase of RP-HPLC. This is not only intuitively reasonable but also reflects an energy-minimized ordered conformation in which an amphipathic arrangement of the peptide's amino acid side-chains has occurred in which all of the hydrophobic residues interact optimally with the lipid-like C_{18} stationary phase while the internal hydrogen bonding expected for α-helices is maintained. Based on these earlier findings, we examined overlapping peptides spanning three different proteins in an effort to correlate their known MHC class II antigenic T cell sites with their RP-HPLC behavior. This approach is based on the assumption that the basic principles of partitioning of a peptide's amino acid side-chain between the solvent and the C_{18} lipid layer of the stationary phase during RP-HPLC are analogous to those involved in peptide–MHC-T cell interactions. Physiological pH conditions were utilized during RP-HPLC for the determination of retention coefficients and retention times.

The most striking finding in this study was that for the three proteins examined all of the peptides which eluted significantly later than predicted corresponded to regions of known T cell helper sites (11 peptides). Peptides 4, 14, 22, and 26, covering the SWM sequence 16–30, 66–80, 106–120 and 126–140, respectively, were found to elute significantly later than predicted (Table I) suggesting that an amphipathic conformation was induced during RP-HPLC for each peptide. In a recent study, a synthetic peptide comprising residues 102–118 of SWM (corresponding to peptide 22, SWM 106–120, from this study) was found by circular dichroism spectroscopy to be 80% helical [in 50% trifluoroethanol (TFE)] and is known to be an immunodominant helper T cell site [27–29]. In the same study SWM 132 146 was found to be 35–40% helical (in 60% TFE). In the current study, peptide 26 (SWM 126–140) shares nine residues with SWM 132–146 and is amphipathic when viewed in a helical wheel array. This region has also been suggested to be an immunodominant T cell site for SWM [28,29]. Myoglobin, however, consists of six amphipathic helical regions folded against one another and the current analysis for this protein may therefore contain an inherent selection bias for amphipathic peptides.

For the CSP, peptides 10, 15, and 16, corresponding to CSP 91–110, CSP 321–340, and CSP 331–350, were found to elute significantly later than their predicted times. Peptides 15 and 16 are found in the polymorphic region of CSP and correspond to a previously determined immunodominant T cell site [24,25]. The peptides chosen to be studied by Good and co-workers [24,25] were selected based on predictive algorithms for amphipathicity and therefore are expected to be biased in the current study [1]. Peptide 10 shares less than half (8 of 20 residues) of known antigenic helper T cell site CSP 103–122 (26), however, peptide 11 (CSP 101–120), which eluted earlier than predicted, contains nearly this entire T cell site. These results suggest that α-helical amphipathicity may not be the sole structural motif responsible for T cell antigenicity. Antigenic helper T cell sites have also been found in earlier studies for CSP 300–310 and CSP 361–380

[25], however, the peptides representing these regions display no anomalous behavior during RP-HPLC. It must also be pointed out that experimental retention times for peptides 6 (CSP 51–70), 8 (CSP 71–90), 9 (81–100), 18 (351–370), and 22 (CSP 391–412) could not be determined in this system due to their extreme hydrophilic nature or poor solubility.

For the HEL peptides 2, 10, 23, and 24, respectively, covering sequences 6–20, 46–60, 111–125, and 116–129, eluted significantly later than predicted (Table II). All of these peptides correspond to reported T cell sites [23]. Since peptides 23 and 24 overlap each other by only 5 amino acids, they are essentially representing only one T cell site. Peptide 10 (46–60) includes the majority of a region of HEL which has been found to be an immunodominant helper T cell site for HEL [30,31]. By helical wheel representation this peptide is amphipathic. Another known immunodominant helper T cell site is HEL 74–86 [32], however, in the current study peptides representing this region of HEL displayed no anomalous behavior by RP-HPLC. HEL is known to have little or no helical nature and is the least likely of the three proteins examined here to contain inherent bias.

The peptides which elute significantly earlier than predicted for each of the proteins studied (Tables I–III) illustrate a markedly different and unclear behavior. One possible explanation for this behavior may be that prior to interaction with the C_{18}, they fold back upon themselves, or become self-associated by hydrophobic interactions, thus exposing only their hydrophilic residues while shielding their hydrophobic residues. This would be expected to effectively decrease the interaction of the hydrophobic residues with the C_{18} and cause the peptide to elute earlier. Additionally, 3 out of the 5 HEL peptides which eluted significantly earlier than predicted correspond to known antigenic helper T cell sites. This also suggests that there may be other conformational arrays (such as the self-interaction of 2 peptides linked by a disulfide bridge) which bind to MHC class II in addition to single amphipathic α-helices.

Every peptide found to elute later than predicted for the three proteins studied contained regions of reported helper T cell sites, and the majority could be configured into an amphipathic array. As found in this study, however, it is evident that amphipathicity may not be the only secondary structural feature governing T cell antigenicity since a number of known helper T cell sites were not confirmed by this method. Those peptides which eluted significantly earlier than predicted, and correspond to known antigenic helper T cell sites, may adopt other structural motifs in their MHC class II interaction. Conformational studies using circular dichromism spectroscopy to determine the secondary structure of those peptides found to exhibit anomalous RP-HPLC behavior would be useful in clarifying alternative structural motifs. If one considers just immunodominant T cell sites for these 3 proteins, 4 out of 7 peptides containing regions of immunodominant T cell sites were found to elute significantly later than their predicted retention times. In conclusion, we have found RP-HPLC to be a useful tool for the study of induced secondary structure of peptides.

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