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Haptenization of Ovalbumin with the Skin Sensitizer Methyl Octanesulfonate: Characterization of the Methylated OVA323-339 T-cell Epitope at His331

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Abstract: Our interest is focused on the induction of allergic contact dermatitis (ACD) by the strong skin sensitizer, methyl octanesulfonate, which is a potent methyl transfer agent, especially to histidine and methionine residues. We are particularly interested to study the effect of methylation on the presentation and recognition of the ovalbumin (OVA) T-cell epitope, OVA323-339, by the T-cell receptor (TCR). Here we report the synthesis of the modified monomer *N*-α-Fmoc-*N*-τ-methyl-L-histidine and its incorporation by solid phase synthesis into the three possible methylated analogues of OVA323-339, that were needed as references for the subsequent studies. Native OVA was haptenized by methyl octanesulfonate. Using classical protein chemistry techniques (trypsin digestion, gel permeation, HPLC, MS and Edman sequencing) we were able to show that OVA323-339 was selectively methylated at His331. Circular dichroism (CD) studies showed that the methylation has no influence on the secondary structure of the peptide. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: allergic contact dermatitis; methyl octanesulfonate; methylated histidine; OVA323-339; ovalbumin; solid phase peptide synthesis; T-cell epitope

INTRODUCTION

The first stage in the induction or elicitation of contact allergy is the interaction of skin allergens or haptens with epidermal proteins. It is now accepted that in the majority of cases, the hapten binds to the protein via a covalent bond [1]. The modified protein is then processed by antigen-presenting cells yielding haptenized peptides that are expressed at the surface of the cell in association with class I or class II major histocompatibility complex molecules (MHC). The complex peptide-MHC is then recognized by a T-cell receptor (TCR) and leads to the immune response [2].

In the past 4 years, our interest has been focused on the induction of allergic contact dermatitis (ACD) by methyl octanesulfonate, a strong skin sensitizer which is a potent methyl transfer agent [3]. The utilization of ^{13}C-enriched methyl octanesulfonate and $\{^1H\}^{13}C$-NMR studies on serum albumin have shown that methionine and histidine residues are preferably methylated [4].

One of our objectives was to study the effect of methylation, which is the minimal chemical modification that can occur, on the presentation to and recognition of the ovalbumin (OVA) T-cell epitope by TCR. OVA323-339 was selected as one of the well known immuno-dominant OVA T-cell epitopes in BALB/c mice (I-A^d) which are particularly susceptible to ACD following sensitization with methyl octanesulfonate [3].

This paper describes the identification of the methylated residue in OVA323-339, in which His328 and His331 are the two potential sites of methylation, after haptenization of native OVA by methyl octanesulfonate.

We also report the synthesis of the modified monomer *N*-α-Fmoc-*N*-τ-methyl-L-histidine and its incorporation by solid phase synthesis into the

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three possible methylated analogues of OVA323-339 that were needed as references. Our data show that OVA323-339 was selectively methylated at His331. The influence of the methylation on the secondary structure of the peptide was studied by circular dichroism (CD).

MATERIALS AND METHODS

The ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM spectrometer, at 200 and 50 MHz, respectively. Chemical shifts (δ) were recorded in ppm and the solvent signal was used as internal standard. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), and m (multiplet). Melting points were determined on a Büchi Tolloti 510 apparatus and are uncorrected. Optical rotations were measured in a 1-dm cell on a Perkin-Elmer polarimeter (Model 241).

ES-MS spectra were recorded on Bio-Q apparatus in the positive mode. MALDI-TOF mass spectra were recorded on a Bruker BIFLEX™ equipped with the SCOUT™ high resolution optics with X-Y multisample probe, a gridless reflector and the HIMAS™ linear detector. A matrix of α-cyano-4-hydroxycinnamic acid (Sigma) was used.

The *N*-terminal sequences of peptides were determined by automated Edman degradation on an Applied Biosystems 473 A microsequencer. Samples were loaded on polybrene-treated and precycled glass-fibre filters. Phenylthiohydantoin-amino-acids were identified by chromatography on a C_{18} column (PTH C-18, 2.1 mm $\times$ 200 mm). PTH-Me-His was separated from PTH-Ala by modification of the eluent A: 18 ml of Premix were added to 1 l of THF 3.5% instead of 15 ml usually used.

CD spectra were recorded on a CD6 Jobin-Yvon dichrograph. The CD data were recorded in a 1-mm quartz cell at 298 K and were expressed in terms of ellipticity units per mole of peptide residues (θ in deg cm^2 dmol^{-1}). Peptides were dissolved in acetate buffer (20 mM, pH 5) and in the presence of TFE (5–80%). The concentrations of peptide samples were calculated according to their net peptide content and were 120 μM.

L-histidine methyl ester dihydrochloride and Fmoc-Arg(Pmc)-Wang resin were purchased from Novabiochem (Meudon, France). *N*-α-Fmoc amino acids derivatives were obtained from Novabiochem and Neosystem Laboratoire (Strasbourg, France), *N*,*N*-dimethylformamide (DMF), *tert*butylmethylether (TBME) and piperidine from SDS (Seltz, France), 1-methyl-2-pyrrolidone (NMP) and triisopropylsilane (TIS) from Fluka (Saint-Quentin Fallavier, France). *N*,*N*-diisopropylethylamine (DIEA) and phenol were purchased from Merck (Darmstadt, Germany), trifluoroacetic acid (TFA) from Acros (Noisy le Grand, France), HPLC grade acetonitrile from Carlo Erba reactif (Nanterre, France) and benzotriazol-1-yl-oxy-tris-(dimethylamino-phosphoniumhexafluorophosphate) (BOP) reagent from Neosystem Laboratoire.

Albumin, chicken egg grade V, was obtained from Sigma (Saint-Quentin Fallavier, France). Trypsin from bovine pancreas (TPCK-treated 52 U mg^{-1} lyophilized) was purchased from Serva (Paris, France). Sephadex G50 coarse was obtained from Pharmacia Biotech (Orsay, France). All other reagents and solvents were of the highest quality commercially available.

Synthesis of *N*-α-Fmoc-*N*-τ-methyl-L-histidine 5

N-τ-methyl-L-histidine dihydrochloride **4** was synthesized as described elsewhere [5], as shown in Scheme 1 in an overall yield of 55%.

To a stirred solution of *N*-τ-methyl-L-histidine dihydrochloride **4** (934 mg, 3.9 mmol) and sodium bicarbonate (976 mg, 11.6 mmol, three equivalents) in a mixture of water (30 ml) and acetone (30 ml) at 0°C was added *N*-(9-fluorenylmethoxycarbonyl)-succinimide (1.95 g, 5.8 mmol, 1.5 equivalents). After being stirred at 0°C for 2 h and at r.t. for 48 h, the mixture was acidified to pH 2 with 6 N HCl and acetone was removed *in vacuo*. The product was taken up in chloroform and washed with 0.1 N HCl and water. The combined organic phases were dried and evaporated *in vacuo*. The residual solid was further purified by chromatography on Sephadex LH 20 (methanol/chloroform, 1/1, v/v) and by HPLC on a RC-Delta Pak C18 column (2.5 $\times$ 10 cm, 15 μm, 300 Å), using a linear gradient from 15% to 80% B over 20 min. Solvent A was H_2O/0.1% TFA and solvent B CH_3CN/H_2O (80/20)/0.09% TFA. *N*-α-Fmoc-*N*-τ-methyl-L-histidine **5** was obtained as a white solid (620 mg, 1.59 mmol, 42% yield).

M.p. = 138–140°C. ^{1}H-NMR (CD_3OD) δ: 3.01–3.25 (m, 2H, CH_2 histidine), 3.79 (s, 3H, CH_3), 4.16 (m, 1H, CH Fmoc), 4.29 (m, 2H, O-CH_2), 4.49 (m, 1H, H_α), 7.25-7.79 (m, 9H, 8H Fmoc + H_b), 8.75 (s, 1H, H_c). ^{13}C-NMR (CD_3OD) δ: 28.2 (CH_2 histidine), 36.2 (CH_3), 48.3 (CH Fmoc), 54.3 (CH_x), 68.1 (O-CH_2), 121.0 (2 $\times$ C IV), 122.3 (CH_b), 126.1 (2 $\times$ CH), 128.2 (2 $\times$ CH), 128.9 (2 $\times$ CH), 132.1 (C IV

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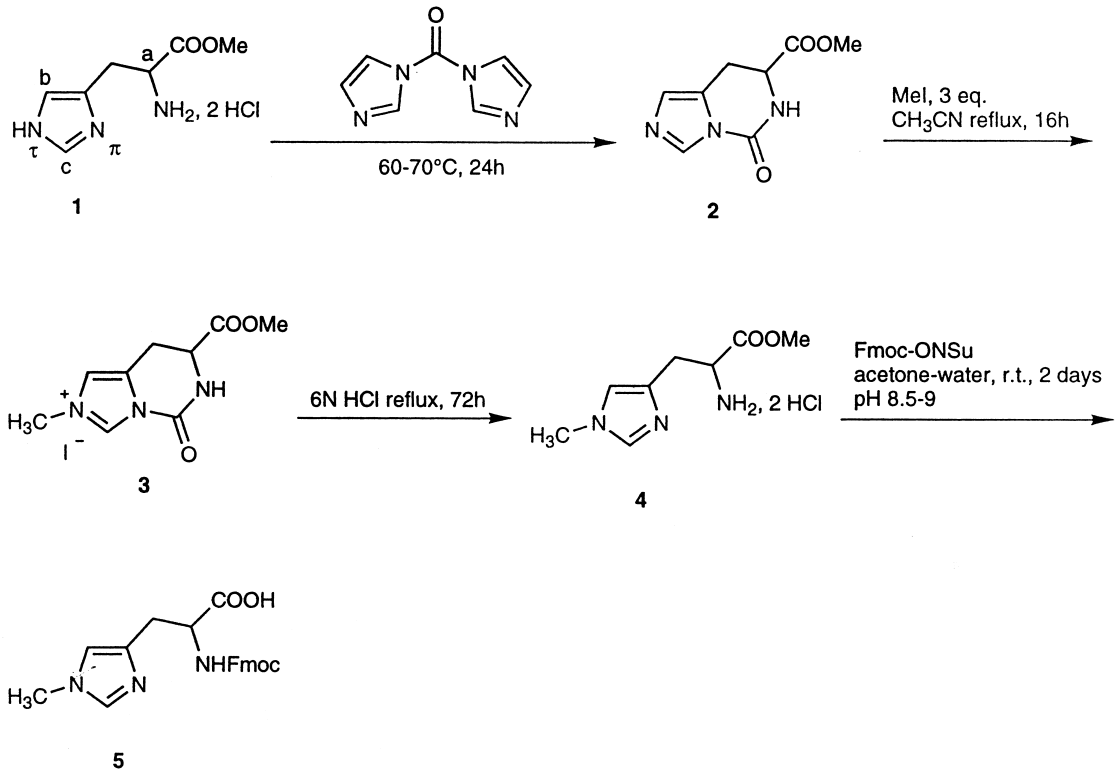

Scheme 1 Synthesis of *N*-α-Fmoc-*N*-τ-methyl-L-histidine.

histidine), 136.5 (CH_c), 142.6 (2 × C IV), 145.2 (2 × C IV), 158.4 (C=O amide), 173.5 (COOH). $[\alpha]_D^{25} = -10.6$ ($c = 1$, CH_3OH). FAB-MS $[M+H]^+$, exp. 392.4, found 392.1.

Peptide Synthesis

Peptides (0.2 mmol) were synthesized manually on a preloaded Wang resin using the Fmoc/*t*-Bu strategy and BOP as coupling reagent. Fmoc amino acids side chain protecting groups were the following: Trt for Asn, His, Gln, *t*-Bu for Glu, Ser and Pmc for Arg. Typically, successive single couplings were performed with three equivalents of Fmoc amino acids and their completion was checked with the Kaiser colour test. A mixture of DMF/NMP (v/v, 1/1) was used as coupling solvent for the last 11 residues. Peptides were cleaved from the resin with the 'low odor mixture' (87.5% TFA, 5% phenol, 5% H_2O, 2.5% TIS), precipitated in TBME and lyophilized after solubilization in 10% acetic acid.

Purification and Characterization of Peptides

Peptides were purified on a radial compression Delta Pak C_{18} column (2.5 × 10 cm, 15 μm, 300 Å), using a Waters HPLC system. Elution was performed at a flow rate of 20 ml min^{-1} with a linear gradient from 5% to 40% B over 20 min. Detection was performed at 214 nm. The crude and purified peptides were analyzed by HPLC on a Delta Pak C_{18} column (3.9 mm × 15 cm, 15 μm, 300 Å). A linear gradient from 10% to 60% B over 20 min was used.

The identity of each peptide was assessed by electrospray ionisation mass spectrometry (ES-MS).

Haptenization of Ovalbumin with Methyl Octanesulfonate

For the purpose of ^{13}C-NMR study (not described in this paper), we used ^{13}C-enriched methyl octanesulfonate for the haptenization of OVA.

^{13}C-enriched methyl octanesulfonate was synthesized as described elsewhere [4]. OVA (150 mg, 3.5

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Table 1 Sequences and Masses of Synthetic OVA323-339 and Methylated Analogues

Peptide	Sequence	Calculated mass	Measured mass
OVA323-339	ISQAVHAAHAEINEAGR	1773.93	1773.78
pMe-His328	ISQAVH(Me)AAHAEINEAGR	1787.96	1787.87
pMe-His331	ISQAVHAAH(Me)AEINEAGR	1787.96	1788.20
pMe-His328/331	ISQAVH(Me)AAH(Me)AEINEAGR	1801.99	1802.06

μmol) was solubilized in 20 ml of 0.1 M phosphate buffer pH 7.4 flushed with nitrogen. ^{13}C-enriched methyl octanesulfonate (154 mg, 0.7 mmol, 200 equivalents) in 1 ml ethanol was then added and the solution was stirred for 7 days at 37°C.

The reagent was removed by dialysis against water at 4°C and by passage through a column of Sephadex G50 coarse equilibrated with 50% HCOOH. The modified protein was recovered by lyophilization.

CNBr Cleavage

Prior to cleavage, OVA was reduced (with DTT) and carboxymethylated (with iodoacetic acid). After lyophilization, reduced and carboxymethylated-ovalbumin (40 mg, 0.9 mmol) was dissolved in 15 ml of 70% formic acid. CNBr (90 mg, 850 mmol, 60-fold molar excess over methionine residues) in 1 ml 70% HCOOH was then added. The sample was kept in the dark, under argon, and at r.t. for 24 h. The sample was diluted about 10-fold with water and then recovered by lyophilization. The cyanogen bromide fragments were separated on a Sephadex G-50 column (1.3 m, 2.3 cm diameter, eluent HCOOH 50%) at a flow rate of 0.2 ml min^{-1}.

Tryptic Digestion

Fraction CN-I (2 mg) was incubated with trypsin (20 μg) for 5 h at 37°C in 0.8 ml of 100 mM Tris–HCl buffer (pH 8). The digestion was stopped by addition of 10% HCOOH. The reaction mixture was then loaded onto an Aquapore C_8 RP-300 HPLC column (250 × 7 mm, 7 μm) and eluted with a linear gradient from 2% to 60% B in 35 min.

RESULTS

Synthesis of *N*-α-Fmoc-*N*-τ-methyl-L-Histidine

Regioselective methylation at *N*-τ position of histidine was obtained as described by Jain [5] after selective protection of the π nitrogen with carbonyldiimidazole (Scheme 1).

In our hands, *N*-(9-fluorenylmethoxycarbonyl)-succinimide was shown to be the more efficient reagent to obtain the *N*-α protected methylated histidine. The identity of the monomer was confirmed by NMR spectroscopy.

Peptide Synthesis

OVA323-339 and its three methylated analogues at histidine residues (Table 1) were synthesized manually on a preloaded Wang resin using the Fmoc/*t*-Bu strategy. No difficulty was encountered in the coupling of methylated histidines. Peptides were purified by RP-HPLC and characterized by ES-MS.

Methylation of OVA with Methyl Octanesulfonate and Characterization of the Modified OVA323-339

OVA was incubated with methyl octanesulfonate (7 days, 37°C) under non-denaturing conditions in a phosphate buffer (pH 7.4). Excess of methylating reagent was removed by dialysis and gel filtration.

Methylated OVA (Me-OVA) was reduced, carboxymethylated and then submitted to CNBr cleavage. The cyanogen bromide fragments were separated on a Sephadex G-50 column (Figure 1).

A similar study run on un-modified OVA showed that fraction CN-I contains the C-terminal CNBr fragment OVA301-385 (data not shown). To obtain

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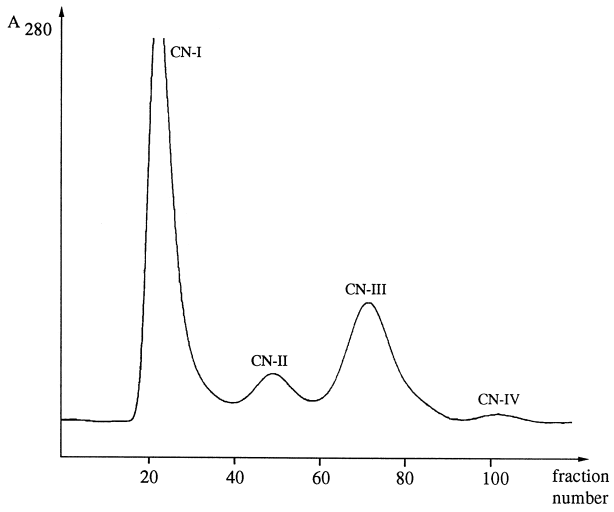

Figure 1 Separation of OVA CNBr fragments on sephadex G-50.

the tryptic peptide OVA323-339, we submitted CN-I to trypsin digestion and analyzed the digest by RP-HPLC (Figure 2). The major peaks were collected and characterized by measurement of their mass by MALDI mass spectrometry.

Peak T-3, which has the same retention time as synthetic OVA323-339, was shown to contain two major peptides: OVA323-339 (M = 1773.85 Da) and a monomethylated derivative (M = 1788.86 Da, ^{13}C labelled; $\Delta M = 15$).

To identify the site of methylation in OVA323-339, peak T-3 was sequenced by Edman degradation. The results were in accordance with the known sequence of the peptide up to residue Ala330. The following residue, His331, was eluted as two PTH-aminoacids: PTH-His and PTH-Ala. The signal corresponding to PTH-Ala was too large to be considered as a residual signal for Ala330.

In order to show that the peak eluted as PTH-Ala did in fact correspond to PTH-Me-His, the synthetic peptide pMe-His328 was submitted to sequencing and Me-His328 was indeed recovered as PTH-Ala. By modifying the HPLC elution conditions (salt concentration) during the automated Edman sequencing of the synthetic peptide pMe-His331 we could indeed detect two separated peaks for PTH-Ala and PTH-Me-His (Figure 3).

Our results show that OVA323-339 was modified by monomethylation at residue His331 and from the MALDI-TOF spectra obtained on peak T-3 we could estimate that methylation occurred to 28%.

Solution Conformation

The CD spectra of OVA323-339 and pMe-His331 in acetate buffer indicate the absence of a well-defined ordered structure. We then examined the CD spectra of both peptides in the presence of TFE (5, 50, 80%). The spectra analysis showed that increasing amounts of TFE induced a α-helical structure characterized by the two negative bands at about 208 and 220 nm (Figure 4). Using the Cdmconv program it was estimated that the α-helix content at 50% TFE was 12% for both peptides.

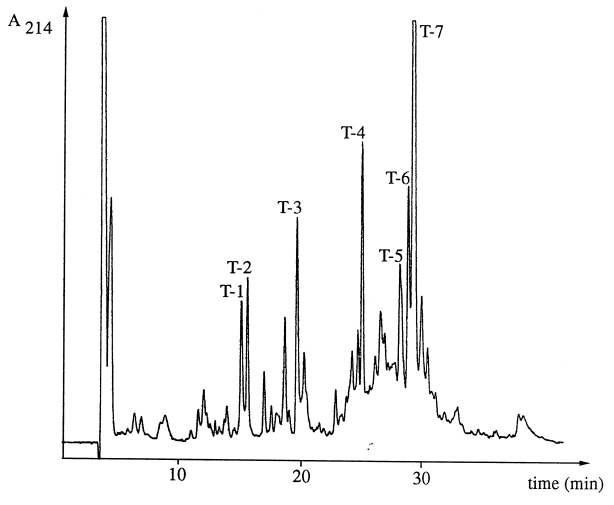

Figure 2 HPLC profile of CN-I tryptic digest.

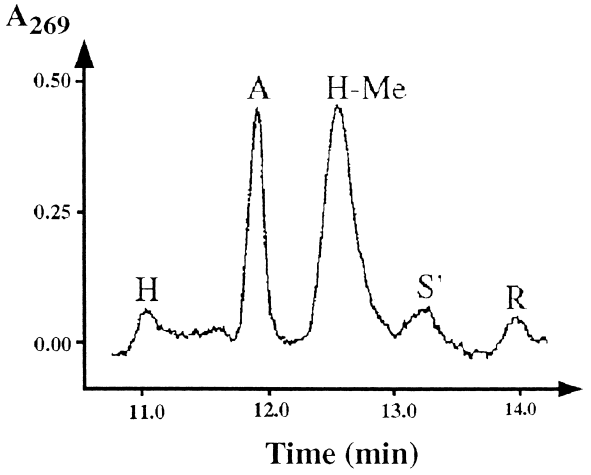

Figure 3 HPLC profile of PTH-Me-His331 on sequencing peptide pMe-His331.

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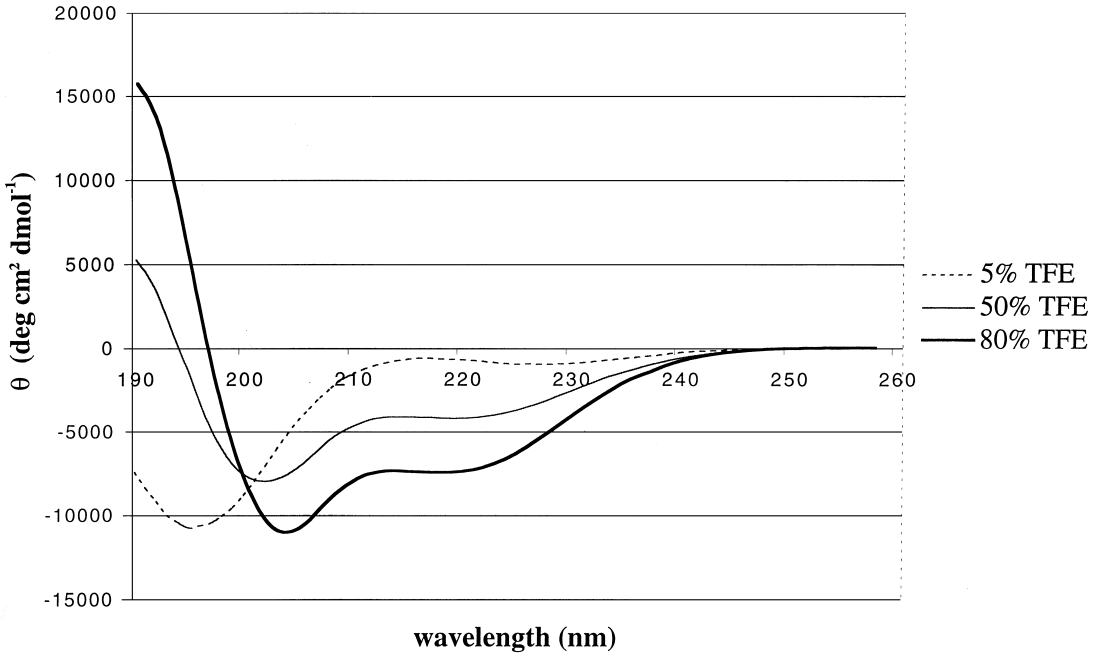

Figure 4 CD spectra of OVA323-339 in 20 mM acetate buffer (pH 5) in the presence of TFE, [peptide] = 120 μm.

DISCUSSION

His328 and His331 are the two potential sites of methylation in the immuno-dominant T-cell epitope OVA323-339 by the skin sensitizer methyl octanesulfonate. In this paper, we have shown that haptenization of native OVA with methyl octanesulfonate results, in the T-cell epitope, in selective methylation at His331. The overall yield of methylation of OVA323-339 was estimated to be 28%.

According to the X-ray structure of OVA [6], OVA323-339 epitope is located at the surface of the protein in an extended conformation. The side chain of His331 is particularly oriented towards the exterior compared to the His328 side chain and forms an hydrogen bond contact with Glu333 which can enhance the nucleophilicity of the N-τ 331. All these structural data can explain the selectivity of methylation we have observed at His331. Other sites of methylation in OVA are under investigation in order to better define the selectivity of haptenization of native OVA by methyl octanesulfonate: indeed we have shown that methionine, and to a lesser extent lysine residues, can also be methylated [4].

We have studied the influence of methylation at His331 on the secondary structure of OVA323-339 using circular dichroism. In 50% TFE, the helix content of OVA323-339 and pMe-His331 was estimated to be 12% for both peptides indicating that methylation, which is the smallest chemical modification that can occur, has no effect on the immunodominant OVA epitope secondary structure in solution.

In the context of ACD, the haptenized peptide is presented to the TCR when complexed with MHC. The recent published crystal structure of OVA323-339/I-A^d complex [7] shows that the peptide is bound to the MHC in an extended type II polyproline conformation and that the two histidine residues, His328 and His331, project out from the MHC-peptide surface and are in excellent positions to be TCR contacts. In order to see if a methylation at His331 of OVA323-339 modifies the conformation of the bound peptide, model studies starting from the known structure of the complex OVA323-339/I-A^d will be investigated for the methylated analogs.

Ise *et al.* [8] have recently shown that the primary response of naive CD_4^+ T-cells derived from transgenic mice expressing the TCR specific for the OVA323-339/I-A^d complex can show distinct activation patterns after stimulation with simple amino acid substituted analogs of OVA323-339. They demonstrated that His328, His331 and Ile334 are essential for the interaction with transgenic TCR in

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OVA23-3 mice. A study of the activity of the three methylated analogs pMe-His328, pMe-His331 and pMe-His328/331 on the activation of naive CD_4^+ T-cells will be useful to elucidate the structural basis for the T-cell response induced by altered peptide ligands (APL) specifically at TCR contact sites. Preliminary results show that OVA323-339 peptide completely loses its ability to induce T cell responses by methylation at His331 (M. Totsuka, personal communication).

Acknowledgements

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REFERENCES

1. Basketter D, Dooms-Goossens A, Karlberg A-T, Lepoittevin J-P. The chemistry of contact allergy: why is a molecule allergenic? *Contact Dermatitis* 1995; **32**: 65–73.
2. Kalish RS. Antigen processing: the gateway to the immune response. *J. Am. Acad. Dermatol.* 1995; **32**: 640–652.
3. Fraginals R, Lepoittevin J-P, Benezra C. Sensitizing capacity of three methyl alkanesulphonates: a murine in vivo and in vitro model of allergic contact dermatitis. *Arch. Dermatol. Res.* 1990; **282**: 455–458.
4. Lepoittevin J-P, Benezra C. ^{13}C-enriched methyl alkanesulfonates: new lipophilic methylating agents for the identification of nucleophilic amino acids of proteins by NMR. *Tetrahedron Lett.* 1992; **33**: 3875–3878.
5. Jain R, Cohen LA. Regiospecific alkylation of histidine and histamine at *N*-1 (τ). *Tetrahedron* 1996; **52**: 5363–5370.
6. Stein PE, Leslie AGW, Finch JT, Carrell RW. Crystal structure of uncleaved ovalbumin at 1.95 Å resolution. *J. Mol. Biol.* 1991; **221**: 941–959.
7. Scott CA, Peterson PA, Teyton L, Wilson IA. Crystal structures of two I-A^d-peptide complexes reveal that high affinity can be achieved without large anchor residues. *Immunity* 1998; **8**: 319–329.
8. Ise W, Totsuka M, Takato R, Hachimura S, Sato T, Ametani A, Kumagai Y, Habu S, Kaminogawa S. Primary response of naive $CD4^+$ T cells to amino acid-substituted analogs of an antigenic peptide can show distinct activation patterns: Th1- and Th2-type cytokine secretion, and helper activity for antibody production without apparent cytokine secretion. *FEBS Lett.* 2000; **465**: 28–33.

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