Analysis of Racemization During "Standard" Solid Phase Peptide Synthesis: A Multicenter Study

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I. Introduction

Synthetic peptides have been produced in abundance by resource and research laboratories for both basic research and drug discovery programs, in numbers estimated in the tens to hundreds of thousands. Inclusion of combinatorial libraries would increase this number to many millions. While improved synthetic procedures and analytical technologies can provide assurance that these peptides have the desired sequence and purity, little or no concern is usually given to stereoisomeric purity. The assumption is often made that racemization is unlikely to occur, or that it need not be examined. The sheer number of peptides which can now be rapidly prepared, either one at a time or in sets or arrays, presents an analytical challenge, whether examining purity or racemization. Yet the critical importance of a unique peptide stereoisomer to its given biological function is widely recognized. Thus, evaluation of the degree to which racemization occurs in peptides produced by core laboratories is timely.

The Peptide Synthesis Research Committee of the Association of Biomolecular Resource Facilities (ABRF) conducts anonymous studies to evaluate the ability of ABRF member laboratories to synthesize and characterize test peptides (1-5). The committee has also conducted studies which provided an opportunity for our member laboratories to attempt new synthetic methods and evaluate new analytical technologies. Previous studies by this committee have shown that peptide assembly and cleavage are no longer significant problems in most core laboratories. Therefore, for its 1996 study, the ABRF Peptide Synthesis Research Committee sought to assess the extent to which racemization occurs during peptide assembly in peptides synthesized by our member resource laboratories.

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The committee prepared for the 1996 study by designing and testing appropriate short peptides that should be straightforward to synthesize. Evaluation of these peptides by the committee also provided the opportunity to establish which tests would be most suitable for identification and quantitation of racemization in this study. During the preparatory phase of this study, it was found that handling and analysis of a hexapeptide with two potential sites of racemization were too problematic to be useful as a model for this multicenter study. A hexapeptide susceptible to racemization at a single His residue was subsequently designed, tested and found suitable for the study. This synthetic peptide was requested from each of the core laboratories for evaluation by the committee. Coded samples of unpurifiedpeptides were characterized by amino acid analysis (AAA), AAA following reaction with Marfey's reagent, high pressure liquid chromatography (HPLC), electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), and enzymatic digestion by carboxypeptidase A (CPA) followed by MALDI-MS. Forty-eight laboratories participated in this study, submitting 53 samples for analysis. In addition to the results of this multicenter study, a summary of the committee's preparatory experiments are also presented in this report.

II. Materials and Methods

ABRF member laboratories were asked to synthesize the following peptide (ABRF96), H-Arg-Glu-Arg-His-Ala-Tyr-OH, by the method most frequently used in their facilities. Crude samples were submitted as dry products equally apportioned in six 1.5-ml microcentrifuge tubes to minimize anyartefacts due to sample handling. Laboratories were requested to provide information on the protecting groups, resin, coupling conditions, and cleavage protocols used. The peptide sequence was chosen to be straightforward to synthesize, with no problems anticipated inpeptide assembly or cleavage. The His residue was included because of its known susceptibility to racemization. Purity of the submitted peptides was assessed by AAA, analytical HPLC, and mass spectrometry. The extent of racemization was evaluated as described below by AAA following reaction with Marfey's reagent, by analytical reversed-phase HPLC, and by mass spectrometric analysis of enzymatic digestion products.

A. Synthesis of Reference Peptides

For both the preliminary and final test peptides, it was necessary to prepare all possible isomers as standards for analysis. These referencepeptides were synthesized using a PE-ABD 430A peptide synthesizer configured for FastMoc synthesis protocols. Piperidine (20%) in N-methylpyrrolidone(NMP) was used to deprotect the N-terminal amino acid. To activate each amino acid in the reaction vessel, the followingwas used: 1 .0 mM amino acid, 1 .0 mM 2-(1 H-benzotriazol-l-yl)- 1 - 1 -2,3, tetramethyluronium hexafluorophosphate (HBTU) in 1 M 1-hydroxybenzotriazole (HOBT) in NMP, 2.0 mM diisopropylethylamine (DIEA). Each His residue was double-coupled.HBTU, DIEA, piperidine, DMF and NMP were purchased from ABI. Methylene chloride was purchased from Burdick and Jackson.

For the preliminary assessment of a hexapeptide with two potential sites for racemization (6,7), the following set of four reference peptides was synthesized:

LL	Arg-Asp-Arg-(L-His)-Glu-(L-Cys)
LD	Arg-Asp-Arg-(L-His)-Glu-(D-Cys)
DL	Arg-Asp-Arg-(D-His)-Glu-(L-Cys)
DD	Arg-Asp-Arg-(D-His)-Glu-(D-Cys)

A second set of reference peptides with one potential site of racemization was subsequently prepared, as shown below:

L	Arg-Glu-Arg-(L-His)-Ala-Tyr
D	Arg-Glu-Arg-(D-His)-Ala-Tyr

For preparation of the reference peptides, all N-a-Fmoc amino acids, Arg (Pmc), Asp (OtBu), Glu (OtBu) and Ala were purchased from ABI/Perkin Elmer (Foster City, CA) in preloaded cartridges. ***D***- or ***L-Cys*** (Trt) 2-chlorotrityl resin was purchased from Anaspec Inc. (San Jose, CA) and the Fmoc-(D- or L-)His (Trt) from Novabiochem (La Jolla, CA). The resin used for the second peptide synthesis was Fmoc-Tyr(tBu)-HMP-resin from Perkin Elmer/ABD.

All reference peptides were cleaved from their respective resins using 500 µl thioanisole, 750 mg phenol, 250 µl 1,2-ethanedithiol, and 500 µl water; the resulting mixtures were diluted to 10 ml total volume with neat TFA. Thioanisole and 1,2-ethanedithiol were purchased from Aldrich (Milwaukee, WI), phenol and ethyl ether from EM Science (Gibbstown, NJ) and acetic acid from Mallinkrodt (Paris, KY). After 2 hr incubation in the cleavage solution, the cleaved peptides were precipitated in -70° ethyl ether, centrifuged, the supernatant decanted, and the precipitation procedure repeated. The final pellet was dissolved in 10 ml 25% acetic acid, lyophilized and redissolved in 0.1% TFA for desalting. All peptides were desalted using a Beckman ODS-RP-C 18 5 µm column, 10 mm x 25 cm, using a O-50% gradient over 60 min: A=0.1% TFA, B = 90% acetonitrile, 0.1% TFA.

B. Amino Acid Analysis

Analyses for amino acid composition were performed according to the method of Spackman, Stein and Moore (8). Samples were hydrolyzed for 24 hr at 11 0°C in 6 N HCl containing 2% phenol and 1% 2-mercaptoethanol. All analyses were performed on a Beckman 6300 amino acid analyzer with a sodium polystyrene sulfonated cation exchange column (Pickering Laboratories).

Determination of ***D***- and L-His content in peptides was carried out using Marfey’s reagent, 1 -fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) (9). Peptides were first hydrolyzed for ***18 hr*** in 6 N HCl under reduced pressure. To ensure that racemization during hydrolysis would be minimized, this time was determined by evaluation of a time course of hydrolysis on the reference peptides. After incubation with 10 mM FDAA in 0.1 M sodium bicarbonate for 1 hr at 40°, the hydrolysates were acidified with 0.2 N HCl before injection on a Hypersil

C 18 column (2.1 x 200 mm, 5µm particle size, 120A pore size). Each hydrolysate was incubated with FDAA immediately prior to analysis. The separations were carried out on a Hewlett Packard HP1 09OM HPLC with a gradient extending from 0.1% aqueous TFA to 60% acetonitrile/0. 1% TFA and detection at 340 nm. The series of test peptides that contained Cys were pyridylethylated before hydrolysis.

C. Optical Rotatory Dispersion

A JASCO model OR-990 chiral detector was tested as a means for assessing racemization in the reference samples. This instrument was equipped with a 44 µl flow cell (25 mm path length), a 150 W Hg-Xe lamp as light source, and an "open-loop" polarizer/analyzer. Samples were analyzed at 350 nm, with a 1 Ox gain, 0.1 sec response, and 16 mdegree deflection. Separations were performed on a Hewlett Packard HP1 090 HPLC with a diode array detector.

D. Analytical Reversed-Phase HPLC

Analytical HPLC of reference peptides included evaluation of two gradient conditions: 1) 0.1% aqueous TFA to 100% acetonitrile/0. 1% TFA, or 2) 0.1 M aqueous ammonium acetate pH 6.5 to 100% acetonitrile/0. 1 M ammonium acetate. Both gradients were run by increasing the acetonitrile concentration by 1% per minute. A Vydac Cl 8 column (4.6 x 250 mm, 5 µm bead, 300 A pore size) was used with both gradients at a flow rate of 1.5 ml/min, and absorbance monitored at 230 nm. An Aztec Cyclobond II-γ chiral cyclodextran-based column was used with gradient 2 under the same elution and detection conditions. Test separations were also performed using a Nucleosil C18 column (4.6 x 250 mm, 5 µm bead, 300 A pore size) eluted with gradient 1 at a flow rate of 0.5 ml/min and detection at 2 14 nm. Analyses of peptides submitted by participating laboratories were routinely carried out on a Hewlett Packard 1090 HPLC using a truncated version of gradient 1 (up to 20% acetonitrile) on the Vydac Cl 8 column at a flow rate of 1.5 ml/min, and absorbance monitored simultaneously at 230 and 274 nm. Exceptional peptides were re-examined using longer gradients.

E. Mass Spectrometry

Electrospray ionization mass spectra were acquired on a Finnigan MAT SSQ700 quadrupole mass spectrometer fitted with an Analytica of Branford electrospray interface. The electrospray energy was -3.5 kV and the nitrogen bath gas was heated to 160 °C. Samples were dissolved in 0.5 ml of 5% aqueous acetic acid and diluted with 50% aqueous acetonitrile/0.5% acetic acid to give a concentration of approximately 15 pmole/µl, based on quantity assessment of a separate sample by amino acid analysis. It is important to note that by this approach, spectral intensity was partially dependent on even sample distribution among the tubes by the submitting laboratory. Analyses were performed by flow injection of 5-µl aliquots, infused at a rate of 1.5 µl/min. Spectral averaging for 1 min was employed prior to profile mode data acquisition. Deconvolution of the ESI mass spectra was accomplished by the BioMass program component of the SSQ data system software.

Liquid chromatography-mass spectrometry (LC-ESI-MS) was performed on a PE-Sciex API-III triple quadrupole mass spectrometer with an IonSpray source. The samples were separated on the Nucleosil C 18 column with gradient 1; a splitting device was used to direct a portion of the effluent into the mass spectrometer. Data were acquired in the range of m/z 400-1000, with sufficient resolution to detect the isotope peaks for singly-charged ions at m/z 1000.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was performed on a Fisons Instruments (Beverly, MA) VGTOfSpec time-of-flight mass spectrometer (0.6 m flight tube) fitted with a nitrogen laser (337 nm, 4 ns pulse). The accelerating voltage was 20 kV and the detector voltage 1.7 kV. Positive ion spectra were collected in the linear mode, with each spectrum derived from the accumulation of 20 to 50 laser shots. External calibrations were performed using synthetic peptides with masses covering the range of interest. Mass accuracy to within 1-2 amu was routinely obtained. Data were analyzed using Fisons Instruments Opus Software.

Upon receipt, peptide samples were dissolved at a concentration of 0.3 pmole/10 µl in 50% acetonitrile/0. 1% TFA and stored at -20°. In order to obtain initial characterization of the submitted peptides, samples were diluted 1: 10 in 50% acetonitrile containing 0.1% TFA saturated with **α-cyano-4-**hydroxycinnamic acid (AC50) as the matrix. In preparation for enzymatic treatment, each peptide solution was diluted 1: 100 with 0.25% ammonium bicarbonate containing 0.5 M NaCl. CPA (Sigma C9762) was diluted 1: 10 from the reagent vial into the above peptide solution. A limit digest was achieved by overnight incubation at 37° Trypsin was also used for some test digestions. For MALDI-MS analysis after enzyme digestion, each reaction mixture was diluted **1: 10** in the AC50 matrix solution. Assessment of component quantity in the peptide samples was obtained by measurement of ion intensities, recognizing the limitations in quantification by MALDI-MS. The calculated average mass-to-charge ratio of the protonated Arg-Glu-Arg-His-Ala-Tyr peptide ($[M+H]^{+1}$) is 83 1.9. The limit carboxypeptidase digest of the L-His containing peptide yields Arg-Glu-Arg with an $[M+H]^{+1}$ at m/z 460.5, while digestion of the D-His containing peptide produces Arg-Glu-Arg-His-Ala, characterized by $[M+H]^{+1}$ at m/z 668.7. The percent racemization was calculated by comparison of the relative peak heights of the m/z 669 and m/z 461 ions in the MALDI mass spectra obtained after CPA treatment of each submitted peptide.

III. Results and Discussion

Analysis Of Reference Peptides With Two Potential Racemization Sites, Arg-Asp-Arg-His-Glu-Cys

The committee first designed a peptide susceptible to racemization at two residues, His and Cys: Arg-Asp-Arg-His-Glu-Cys. Glu was placed after the Cys in order to facilitate determination of the influence of a racemized cysteine on the action of CPA. Arg was placed after the histidine in order to permit examination of whether the presence of a D-His would stop the action of trypsin.

The committee's first attempts to identify methods to detect and distinguish among the four isomeric peptides focused on HPLC. In these preliminary studies, it was found that reversed-phase HPLC was readily able to separate the four possibleisomeric forms of the referencepeptide Of the several separation systems evaluated, the Nucleosil column (described above) provided the best resolution while the cyclodextran-based chiral column was unable to separate the four reference peptides. However, a major problem was encountered in that there was a noticeable change in the number of peaks in the HPLC profile of each "pure" peptide when rechromatographed after a period of time. These changes could not entirely be attributed to oxidation of sulfhydryl groups to disulfides. A further difficulty was found in evaluating racemization in these peptides by AAA following reaction with Marfey's reagent. Although there was satisfactory separation of derivatized ***D***- and L-His, ***D***- and ***L-Cys*** were incompletely resolved, seriously compromising accurate quantification. As a consequence, analysis of the four referencepeptides by this technique showed variable amounts of Cys racemization that were inconsistent with the behavior of the purified peptides on HPLC. It was not clear if racemization was occurring during the analysis process per se. There were also complications in interpreting the results of MALDI-MS analysis after CPA digestion of the four reference isomeric peptides due to variations in the proteolytic product distribution. Thus, in view of the fact that the problems already encountered with peptide characterization would be expected to be exacerbated for the samples submitted from member laboratories because of the additional steps in shipping and handling, this peptide model was deemed unsuitable for the purposes of the 1996 study.

Analysis Of Reference Peptides With A Single Potential Site of Racemization, Arg-Glu-Arg-His-Ala-Tyr

A simpler peptide that was susceptible to racemization at only a histidine residue was prepared: Arg-Glu-Arg-His-Ala-Tyr. Of the HPLC separation protocols tested, only the chiral column was ineffective in resolving ***D***- or L-His containing peptides. The others achieved baseline resolution (Figure 1A). Interestingly, the order of elution of the isomers was reversed when ammonium acetate was used in the mobile phase (gradient 2) instead of TFA (gradient 1). During AAA following reaction with Marfey's reagent, the D-His and L-His derivatives were well resolved, with a 2.1% racemization detected for the L-His-containing peptide, and 1.9% detected for the D-His-containing peptide.

Direct analysis with the on-line ORD detector linked to the HPLC was hampered by peak broadening, which led to incomplete resolution of the two peptides. Diffusion in the large flow cell may be a major factor in this difficulty. Because detector response can be either positive or negative, it is important to have an excellent signal-to-noise ratio and baseline HPLC resolution of the peptide isomers to be able to quantitate the degree of racemization by this technique. While the on-line ORD detector is promising, further improvements in resolution as well as sensitivity are required for it to be useful for routine peptide analysis.

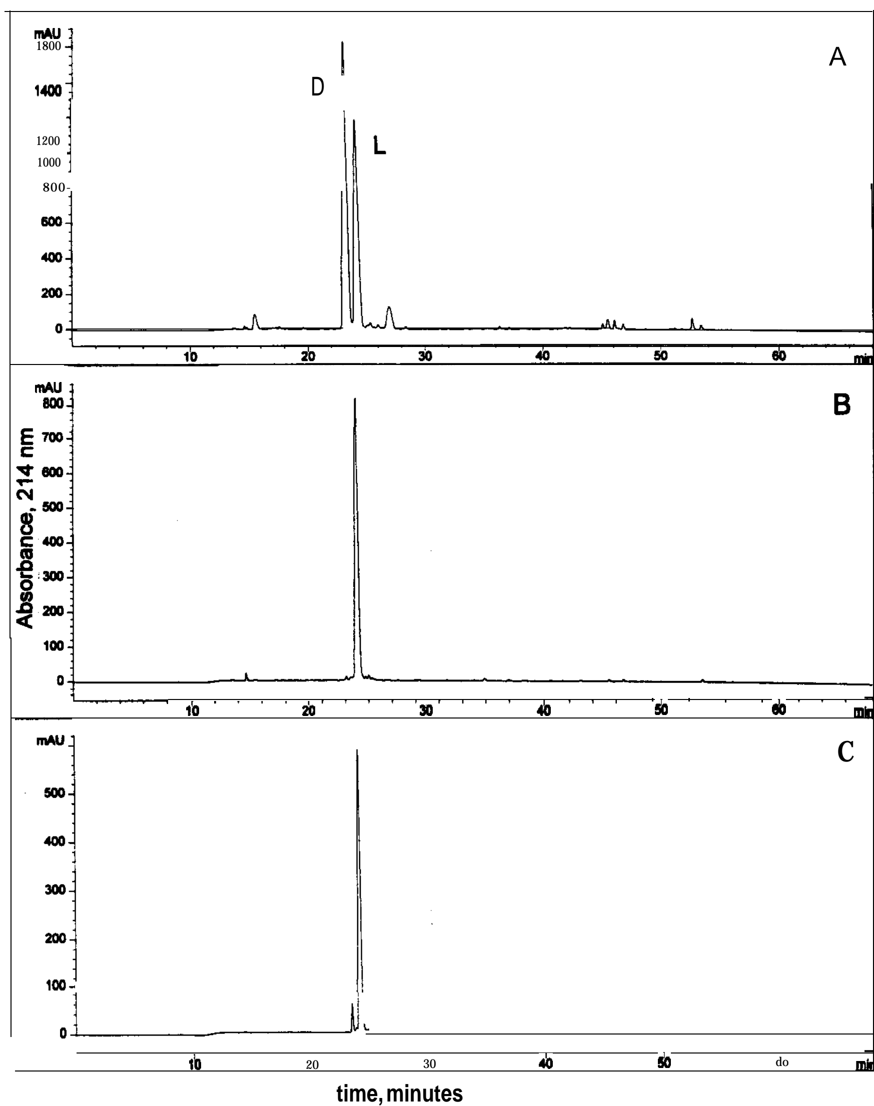

Figure 1. HPLC Profiles of Reference Peptides and Selected Samples
A = mixture of D and L reference peptides; B = sample 9050; C = sample 5 110

Table 1. Peptide Evaluation Sheet

SAMPLE #	Chemistry (Fmoc default)	Protecting group (Trt default)	% desired HPLC	% desired MALDIMS	% desired MALDIMS, diluted	% D Marfey's	% D HPLC	% D CPA/MS	other molecular species present by ESI-MS or MALDI-MS analysis
175		Bom	70	69		2.58	3.34	nd	some +12
484			90	85		1.91	3.98	nd	trace +Pbf
690			94	88		2.18	0.46	nd	trace +Fmoc
988			81	71		3.12	1.25	nd	
1002			78	68		5.32	4.31	4.1	some +Tyr
1295			87	88		5.69	5.99	5.9	
1354			95	63	94	2.26	3.25	nd	trace +Pmc
1609			87	79		4.85	4.85	nd	trace +Tyr, +Fmoc, +tBu, +71
2522	Boc	Dnp	86	77		2.57	1.59	nd	trace +Bzl
3845		Boc	93	96		3.83	5.30	nd	
4088			98	96		2.02	1.14	nd	
4308			70	65		5.18	6.97	nd	trace +Tyr, +Fmoc
4343			98	93		2.27	1.69	5.7	
4609			94	88		3.00	2.28	nd	
4612			60	69		2.49	7.68	1.4	some -Arg, trace +Tyr, +Pbf, +Pbf & tBu
4761			97	93		2.03	3.25	nd	
5110			94	96		7.15	5.76	21	
5111			94	98		7.22	5.53	14.9	
5112			94	98		7.47	5.81	10.1	
5113			83	97		6.99	5.52	15.7	
5114			94	98		6.75	5.48	8.6	
5307			95	86		2.06	2.16	nd	trace +Pbf
5874			98	56	91	2.24	1.59	nd	some +Pmc
6410			0	18		4.57	nd	nd	failed synthesis
6849			97	82		2.25	1.61	nd	trace +Pmc, +224
6930			70	84		1.93	3.37	nd	trace Orn substitution
7154			91	41	92	2.67	2.11	nd	trace +Pbf
7325			98	94		2.31	1.97	nd	
7440			84	80		3.96	3.14	nd	some +Arg, +Pbf
7638			90	93		3.09	5.66	2.6	
7818			0	0		6.16	1.66	nd	Arg-Gly-Arg-His-Ala-Tyr
8246			60	76	70	2.88	0.53	nd	significant -Arg
8398			99	97		1.96	0.06	nd	
8402			95	68	84	4.62	4.57	nd	trace +Tyr, +Fmoc
8409			93	78		4.87	6.30	10.3	some +Pmc
8422			92	83		7.07	4.67	5.1	trace +Pmc
8465			56	36		2.72	1.12	nd	significant +nTyr, up to n=7
8475			56	43		2.82	1.42	nd	significant +nTyr, up to n=7
8583			100	96		2.14	0.04	2.9	
8596			96	76	81	2.53	3.09	nd	trace -22, +Pmc
8808			100	97		2.20	0.00	nd	
9050			100	98		2.26	0.00	2.8	
9140			94	77	97	8.11	5.62	17.6	trace +Fmoc
9280			98	89		3.65	0.18	nd	trace +Pmc
9453	Boc	Dnp	98	80		1.91	0.10	nd	trace +anisyl, +BrZ
9818		Mtt	85	68	81	6.12	7.09	2.9	some +Tyr, trace +Pbf
DBE1			87	94		3.63	12.96	nd	some -His
FBR1			88	90		5.25	4.18	5.7	trace +Tyr
JL96			89	79		2.67	1.56	nd	trace +112, +Pmc
ND05			94	97		2.53	1.55	nd	
PEPA			84	86		2.58	1.63	nd	some +Tyr
PEPB			89	92		na	3.78	nd	

na = not analyzed. The hydrolysate of PEPB precipitated after reaction with Marfey's reagent

nd = not detected

The usefulness of combining MALDI-MS with proteolytic digestion to provide insight into the extent of racemization was found to depend on the enzyme used for digestion. Trypsin did not discriminate between peptides with D- and L-His adjacent to the cleavage site. However, CPA proved very sensitive to the presence of D-His, even distal to the cleavage site. As seen in Figures 2A-D, CPA removed only the terminal Tyr residue from the D-His-containing peptide, leading to a characteristic ion at *m/z* 669 (Arg-Glu-Arg-His-Ala) instead of the *m/z* 461 ion (Arg-Glu-Arg) observed for the L-His-containing peptide. Although this particular assay may not be of general utility, similar strategies could most likely be designed for other sequences.

Determination of Purity of Coded Samples by Different Analytical Methods

Fifty-three peptide samples were submitted by 48 laboratories. Previous studies by this committee have demonstrated the need for multiple analytical methods for the assessment of purity. Therefore the peptides in this study were analyzed by AAA, HPLC, ESI-MS and MALDI-MS to determine purity (Table I). Only two peptide samples had less than 50% of the desired product, and three other samples had less than 70% of the desired product, as judged by their mass spectra, amino acid composition and HPLC retention time. Overall, the peptides were of excellent quality.

In assessing peptide purity, there were occasional discrepancies noted among the different analytical methods. MALDI-MS analysis of the peptide samples often revealed impurities not readily observed by ESI-MS and HPLC. In most of these cases, greater dilution of the sample prior to MALDI-MS yielded data more consistent with ESI-MS and HPLC assessment. This is illustrated by sample 1354 in Figure 3A-C and in Table I. These results underscore the importance of using more than one analytical method to evaluate peptide purity, and of testing samples under several different conditions.

Analysis of Coded Study Samples for Extent of Racemization

Racemization was judged by three methods: AAA following reaction with Marfey's reagent, HPLC, and MALDI-MS of products produced by digestion with CPA. Although most samples that were chemically pure were found to have low levels of racemization, a few very pure samples showed extensive racemization. For example, AAA, HPLC, ESI-MS (data not shown) and MALDI-MS analysis (Figure 2E) of sample 5 110 revealed that this sample contained a high percentage of the correct sequence. However, both CPA/MALDI-MS (Figure 2F) and HPLC analysis (Figure 1 C) of 5 110 revealed high levels of the D-His-containing product as compared to sample 9050 (Figure 1B), which showed little racemization by all criteria used. AAA following reaction with Marfey's reagent was consistent with these data.

There was generally agreement between the results from determination of racemization by AAA following reaction with Marfey's reagent, by HPLC, with somewhat less agreement by CPA/MALDI-MS. However, in some cases the three analytical methods yielded divergent results. Therefore, the possible sources of discrepancy among the methods were also evaluated as part of this

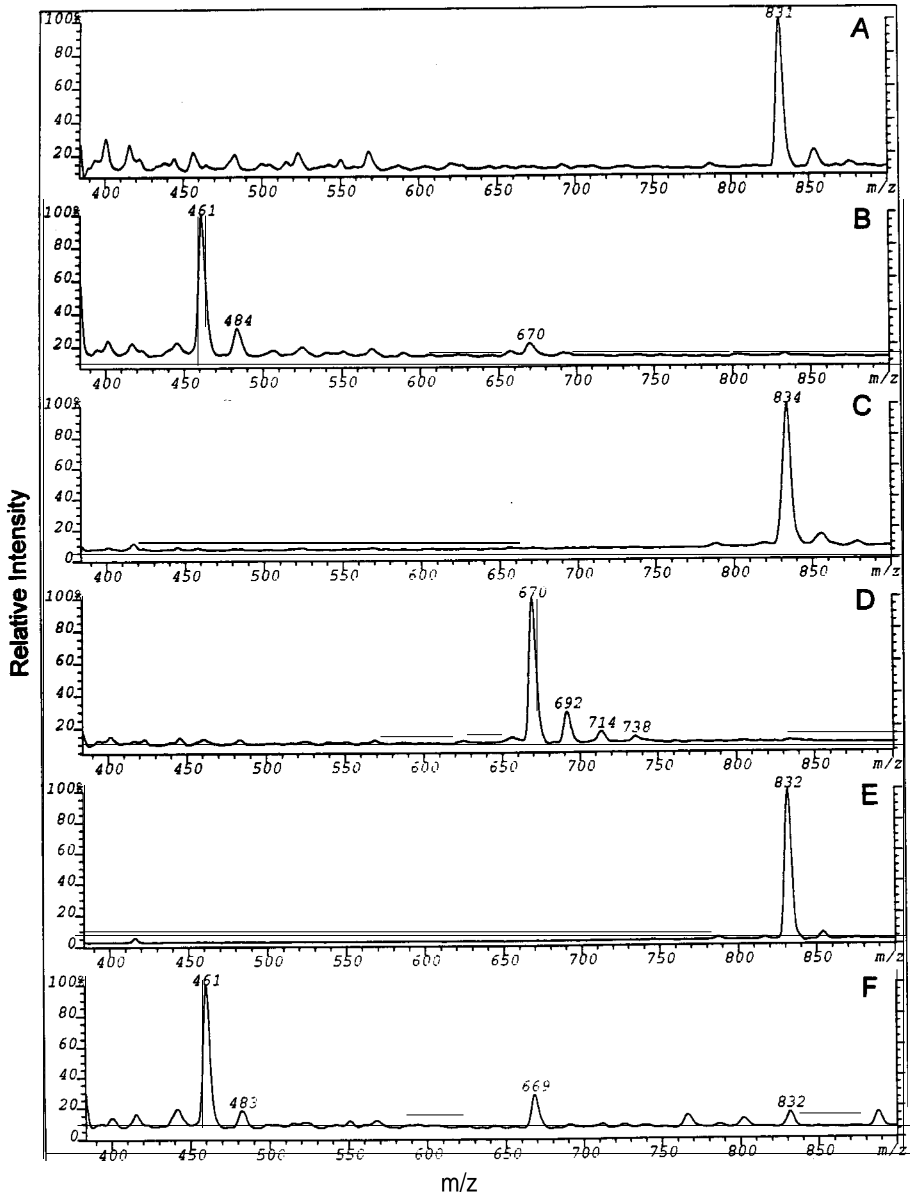

Figure 2. MALDI-MS Analysis of Peptides Before and After Treatment with CPA
A, C and E: samples analyzed in the absence of CPA; B, D and F: samples analyzed after treatment with CPA; A and B: L-reference peptide; C and D: D-reference peptide; E and F: sample 5110

study, CPA/MALDI-MS analysis tended to detect the presence of D-His only in those cases where there was high racemization. When the percentage of D-His-peptide estimated by HPLC was found to be very low, the small peaks present were not recognized by the instrument's computerized integration protocol.

In several instances, AAA following reaction with Marfey's reagent revealed higher racemization than shown by either HPLC or CPA/MALDI-MS; this most frequently occurred when there was a failed synthesis, such as samples 6410 and 78 18 (see below). Information can be obtained from AAA following reaction with Marfey's reagent even if the synthesis fails. In contrast, HPLC and CPA/MALDI-MS are more dependent on the presence of the correct sequence. In order to effectively utilize the HPLC and CPA/MALDI-MS methods with less pure samples, the corresponding signature ions and retention times of the modified products must be ascertained.

For several other samples, the percentages of D-His-containing peptide determined by HPLC were higher than by AAA following reaction with Marfey's reagent or by CPA/MALDI-MS. For example, this discrepancy was observed for sample 4612, which contained a significant amount of des-Arg peptide, and for sample DBEl , which contained some des-His peptide. A possible explanation for this is that the D-His-peptide elutes just before the L-His-peptide in the HPLC analysis. Peptide byproducts in which basic residues have been deleted would be expected to shift to an earlier retention time, perhaps coeluting with the D-His-containing peptide.

Figure 4 shows the distribution of the results from AAA following reaction with Marfey's reagent. Because of the skewed distribution of data, assessment of the mean and/or median extent of racemization was judged not to be useful. The majority of samples contained approximately 2% of the D-His form of the peptide It should be noted that of the ten samples with the highest extent of racemization, five comprised the 5 110-5 114 series from one laboratory. Racemization of histidine is attributed to a base-sensitive, intramolecular reaction which occurs at the imidazole n-nitrogen(6,l0). This reaction is influenced by the type of base used in the coupling step of solid phase peptide synthesis, by the relative strength of the coupling reagent, and by the polarity of the solvent. In order to control the extent of racemization, different side chain protecting strategies have been devised for both the - and τ-imidazole nitrogens. All but five of the samples submitted for this study used a Trt group on the T-nitrogen (Table I), an approach reported to be effective in suppressing racemization. The results of the study suggest that the Trt group is not always as effective as desired. Five samples were produced by other protecting strategies, but this is an insufficient number to permit statistical evaluation of the effects of these groups. The two peptide samples synthesized by Boc chemistry(2522,9453) both used Dnp-protected His, and had low levels of racemization. Peptide 98 18 used Mtt protection and had a high content of D-His-containing peptide. One sample (175), which used Born protection of the n-nitrogen, showed the presence of a +12 amu derivative by MALDI-MS. This modification is observed frequently as the result of reaction of formaldehyde released by the Born group during cleavage procedures. Sample 3845 used Boc protection of His, but had elevated levels of D-His peptide.

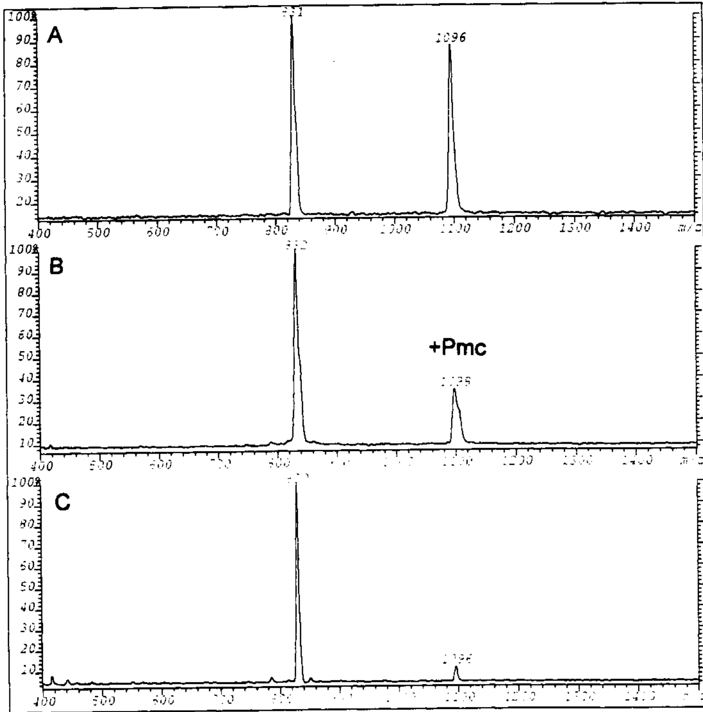

Figure 3. Effect of Sample Dilution on MALDI-MS Analysis of Sample 1354
A: 300 pmoles; B: 30 pmoles; C: 3 pmoles

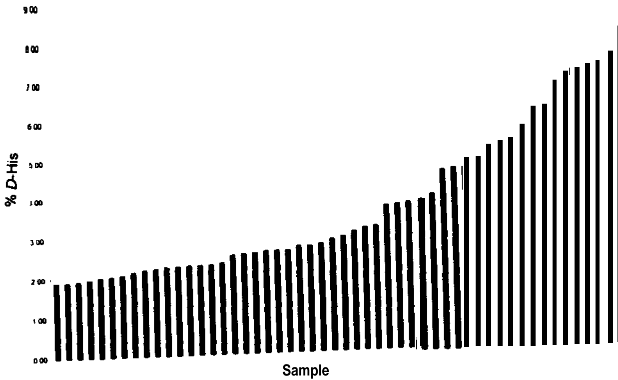

Figure 4. Distribution of Data from AAA Followed by Marfey's Analysis

Samples 5110-5114, submitted by one laboratory, were all excellent in chemical purity, but lower in optical purity. The preparation of these five samples differed only in the length of coupling time. Examination of the detailed protocol sheets submitted by participating laboratories revealed that there was no correlation between coupling times and extent of racemization among the samples submitted, even though a wide variety of coupling times had been employed. Similarly, no correlation was noted for the type of base used in the coupling cocktail. Out of concern that the amount of D-His-containing peptide might simply reflect the quality of the starting protected His derivative used in the synthesis, additional information about the vendor and lot number of the compound was requested anonymously from the study participants. No correlation was found between the extent of racemization and the vendor of the L-His derivative. There were five pairs of peptides for which each sample was synthesized using the same lot of His derivative. Two pairs of peptides synthesized with the same lot of protected His derivative were in the cohort of samples with low racemization. It was interesting to note that another pair had one member with low racemization and the other with approximately 4-5% of D-His-containing peptide. Two other peptide pairs had one sample with low racemization, and the second with among the highest racemization observed. Thus, it appeared unlikely that the origin of the racemization could be the quality of the starting material.

Analysis of Synthetic Failures

From the studies conducted by this committee in the past several years, it can be concluded that the quality of peptides produced by ABRF member laboratories is high, as judged by modern analytical techniques. However, there have always been a few less than satisfactory samples for which the source of synthetic problems could only be attributed to human error. In the present study, errors of this type originated not only from some member laboratories, but from manufacturers, as illustrated in some of the examples described below.

Sample 6410 appeared to be a failed synthesis, with little detectable peptide Sample 7818 was quite pure, but had no correct product, since Gly had been substituted for Glu in the synthesis. The laboratory submitting sample 7818 used an instrument with amino acid cartridges that were refilled with bulk amino acid derivatives. Although it is not known whether the refilling procedure was carried out in-house or by a commercial supplier, this type of opportunity for a mistake could affect many peptide syntheses, and should be a cause for concern in all laboratories. Sample 8246 contained a large quantity of peptide missing the C-terminal Tyr. From the description on the protocol sheet, it appeared likely that this laboratory had prepared its own Tyr-resin. Yet purchase of preloaded resins provides no assurance of quality.

Analysis of the samples by MALDI-MS revealed the presence of additional Tyr residues on ten peptide samples, nine laboratories. As noted above, at higher analyte concentrations, MALDI-MS frequently shows greater intensity for by-product ions than appropriate for their concentration in the sample. Indeed, ESI-MS analysis of the samples introduced by flow injection revealed only a few instances of samples with ions corresponding to additional

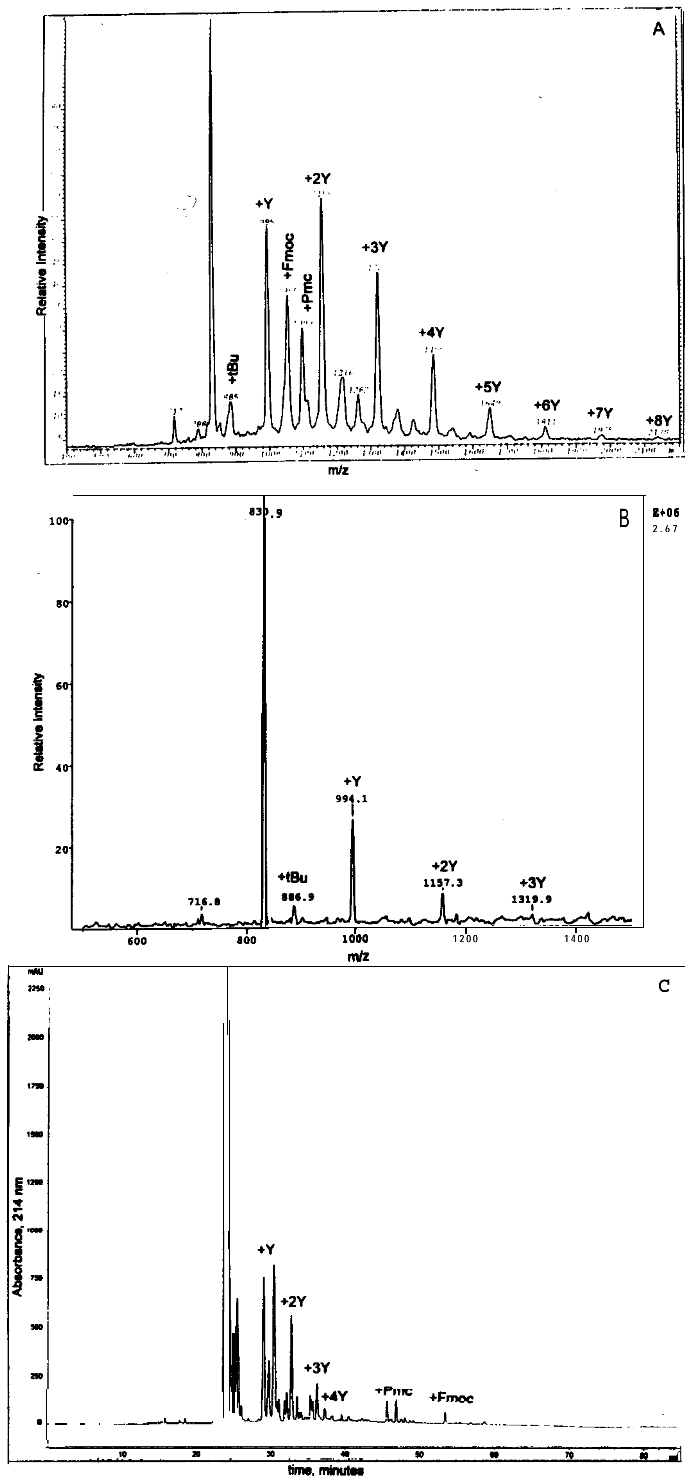

Figure 5. Analysis of Sample 8475
A = MALDI-MS; B = ESI-MS; C = HPLC

Tyr residues. LC-ESI-MS analysis of sample 8475 permitted detection of peptides containing up to 7 additional Tyr residues. Consistent with this observation, the amount of Tyr detected by AAA was very high. The MALDI-MS and ESI-MS analyses of this sample are shown in Figure 5A-B. HPLC analysis of all ten +Tyr-containing samples showed that seven had less than 2% of the +Tyr peptide. Three samples, including 8475 (Figure 5C), had significant amounts of the +Tyr peptide and other peptides containing a variable number of Tyr residues. The laboratories which submitted all ten of these samples had all indicated that they had purchased resin with the Tyr already attached. However, these resins appeared to be purchased from several vendors. Thus, additional quality control of resins sold by manufacturers should be implemented.

IV. Conclusions

The overall results from this study indicate that racemization during peptide assembly is not a serious problem in most participating laboratories. Nevertheless, it is evident that laboratories can produce peptides which have predominantly composed of the desired product, yet exhibit unacceptably high levels of racemization. AAA following reaction with Marfey's reagent permits very sensitive detection of racemization, and complementary procedures involving enzymatic digestion coupled to MS detection can also be devised. Unfortunately, HPLC separations for AAA following reaction with Marfey's reagent do not always provide baseline separation of pairs of ***D***- and L-amino acids. Moreover, in laboratories which produce large numbers of peptides, the additional personnel and instrument time required to analyze all peptides by this method may preclude routine use of this method. Nonetheless, in the present study, it was possible to achieve baseline resolution of the ***D***- and L-His containing study peptides by conventional reversed-phase HPLC chromatography. Since fortuitous separation of racemized peptides by HPLC cannot be assumed, accurate determination of racemization should be considered for peptides which are central to large research programs.

Considerable thought and emphasis should be placed on the potential effects of a racemized product on the outcome of a project. It is well known that the ***L*** and ***D*** form of peptides can vary significantly with respect to stability and biological activity. Therefore, for example, if a significant portion of a synthesis is racemized and the racemized peptide is 1000 times more stable in vivo, the majority of the immune response may be directed to the racemized peptide and not to the desired peptide. This could be one explanation for peptide immunizations which result in antisera of suboptimal avidity or specificity. Racemization could also be an explanation as to why positive results obtained with a peptide library cannot be repeated with the newly synthesized, highly purified product. The implications of the above examples require serious reflection by the investigators designing the experiments and by the core laboratories which supply the requested peptides.

In most of the previous studies conducted by this committee, a few peptides stood apart from the majority of high quality peptides as samples whose problems originated from human error. However, in the present study it was

clear that these errors were not limited to the participating peptide resource laboratories, but extended perhaps to a greater extent to reagent suppliers. While three samples exhibited serious problems with peptide assembly in the participating laboratories, nine laboratories had purchased faulty preloaded resins. The critical role of peptides in research programs requires that both suppliers and core synthesis laboratories examine their quality control procedures. While strict application of GLP or IS09002 protocols may not be necessary for many laboratories, incorporation of some of the procedures into general laboratory operations might be considered in order to provide additional assurance that high laboratory standards are maintained, and that errors, when they occur, will be detected sufficiently early to prevent unnecessary expenditure of time and effort, and loss of good will.

Although the results of this study cannot be used to formulate recommendations about remedies or precautions to be taken, they do emphasize the importance of seriously considering analysis of racemizationIn the present study, it was not possible to identify the the origins of high levels of racemization that were occasionally found inpeptides from laboratories whose instruments and personnel seemed to be otherwise working at high performance levels. While the length of coupling times, the type of base, or the source of the amino acid derivatives did not appear to be a factor in the racemization detected in this study, it is possible that the purity or age of coupling reagents could be important, since it has been demonstrated that His racemization takes place in the coupling step. Synthesis of the reference peptide sequence used in this study may be a helpful approach to determine whether coupling conditions are optimized, since the *D*-His and L-His forms of this peptide are so easy to separate by HPLC.

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