*Proc. Natl. Acad. Sci. USA*
Vol. 89, pp. 5630–5634, June 1992
Biochemistry

# Protein surface topology-probing by selective chemical modification and mass spectrometric peptide mapping

[plasma desorption mass spectrometry/hen-egg white lysozyme/lysine acetylation/$N^7$,$N^8$-(dihydroxy-1,2-cyclohexylidene)arginine/surface accessibility]

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*Communicated by Fred W. McLafferty, February 19, 1992 (received for review September 27, 1991)*

**ABSTRACT** **Aminoacetylation of lysine residues and the modification of arginine by 1,2-cyclohexanedione to $N^7$,$N^8$-(dihydroxy-1,2-cyclohexylidene)arginine were used for probing the surface topology of hen-eggwhite lysozyme as a model protein. The molecular identification of lysine and arginine modification sites was provided by molecular weight determinations of modified and unmodified tryptic peptide mixtures (peptide mapping) using $^{252}$Cf plasma desorption mass spectrometry. At conditions of limited chemical modification, mass-spectrometric peptide-mapping analyses of lysozyme derivatives enabled the direct assignment of relative reactivities of lysine and arginine residues at different reaction times and reagent concentrations. The relative reactivities of lysine residues showed a direct correlation with their surface accessibilities from x-ray structure data. For the reaction with 1,2-cyclohexanedione, a selective modification at Arg-5, -125, -112, and -73 was identified, and an inverse correlation of relative reactivities with the surface accessibility ratios of the $N^7$- and the $N^8$-guanidino functions was obtained. By examination of the x-ray structural data of lysozyme, this selective modification was attributed to intramolecular catalysis because of the presence of neighboring proton acceptor groups, such as the Asp-119 carboxylate group for Arg-125 and the Trp-123 and Arg-125 carbonyl groups for Arg-5.**

While our understanding of the structural properties of proteins and their mobility in the crystalline state and in solution has improved rapidly in the last years because of x-ray analysis (1, 2), NMR methods (3, 4), and simulation approaches (5), structural data have been correlated only rarely with the chemical properties of individual amino acid residues. Chemical reactivities of amino acid residues have been mainly determined by competitive labeling techniques, which, however, are laborious and generally require radioactive probes (6). For example, acetylation of amino groups has been extensively used for modifying enzymatic properties, immunological reactivity, and proteolytic digestion patterns (7). Incomplete acylation of proteins has been frequently observed (8), while free accessibility of reactive residues is generally anticipated (7). In spite of some success of this view [e.g., in differential labeling studies of free and antibody-bound antigens (9)], parameters like electrostatic interactions and other microenvironmental effects may obscure a direct understanding of amino acid reactivities (6). Thus, in addition to an accurate description of protein structure and dynamics, experimental approaches that rapidly provide detailed chemical reactivity data are needed in structure–function studies of proteins.

The development of "soft ionization" techniques of mass spectrometry, such as fast atom bombardment (FAB), $^{252}$Cf plasma desorption (PD), and more recently electrospray (ES), has enabled accurate molecular weight determinations of polypeptides and proteins (10–12). A powerful approach in recent work is the direct mass spectrometric analysis of proteolytic digestion mixtures (peptide mapping), which has been applied to primary structure studies such as the characterization of cDNA-derived sequences, and the identification of posttranslational modifications (10, 13, 14). Mass spectrometric peptide mapping enables the simultaneous identification of proteolytic peptides even in complex mixtures and, therefore, is well suited for screening modified amino acid residues in chemical reactivity studies of proteins.

In this study peptide mapping by PDMS has been applied to the assignment of relative reactivities of lysine residues toward acetylation and of arginine groups toward 1,2-cyclohexanedione (CHD) under mild conditions of limited chemical modification. Hen-eggwhite lysozyme (HEL) was used as a model protein because extensive structural information (5) enabled a direct comparison with chemical reactivity data. In contrast to the aminoacetylation, the reaction of arginine residues to form $N^7$,$N^8$-(dihydroxy-1,2-cyclohexylidene)arginine (DHCH-Arg) has been used rarely but appeared interesting—e.g., for the reversible modification of trypsin substrate sites (15); in particular, the poor reactivity of arginine residues in small peptides (16) compared with proteins (17) suggested tertiary structure-related reactivity parameters beyond (for instance) a high surface accessibility (18). For both reactions, chemical reactivity data were obtained that show mass spectrometric peptide mapping to be an efficient approach for probing protein surface topology and identifying the selectivity of modification reactions.

## MATERIALS AND METHODS

**Preparation of Aminoacetylated Lysozyme Derivatives (Ac-HEL).** Acetylation of lysine and N-terminal amino groups was essentially performed as described (8). Acetic anhydride, analytical grade (Riedel de Haen, Seelze, F.R.G.) at various concentrations (10–10,000 mol/mol of amino group) was added to 1 ml of 0.7 mM (10 $\mu$g/$\mu$l) HEL (Fluka) in 0.5 M $NH_4HCO_3$. Reactions were carried out at pH 7 (maintained by addition of 25% $NH_3$) for 30 min at 20°C, and products were lyophilized and redissolved in 0.1% trifluoroacetic acid.

**Preparation of DHCH-Arg-Containing Lysozyme Derivatives (DHCH-HEL).** Modification of arginine residues was carried out as described (15, 17). CHD (Merck; 33- and 100-fold molar exess per mol of arginine) was added to a 0.14

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Abbreviations: PD, $^{252}$Cf plasma desorption; ES, electrospray; PDMS and ESMS, PD and ES mass spectrometry; HEL, hen-eggwhite lysozyme; Ac-HEL, acetylated HEL; CHD, 1,2-cyclohexanedione; DHCH-Arg, $N^7$,$N^8$-(dihydroxy-1,2-cyclohexylidene)arginine; DHCH-HEL, HEL containing DHCH-Arg; SA, surface accessibility; NC, nitrocellulose.

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mM solution (80 μg) of HEL in 40 μl of 0.2 M sodium borate (pH 9) (50 and 150 mM CHD, respectively). Reactions were carried out for 120 min at 37°C, and aliquots (15 μg) were withdrawn at various times (see Fig. 2*b*). Reactions were terminated by cooling to 0°C and adding 5 μl of 50% acetic acid; products were analyzed immediately by PDMS.

**Mass Spectrometry.** Nitrocellulose (NC) surfaces for sample adsorption in PDMS were prepared by electrospraying (19). HEL derivatives (15 μg) were allowed to adsorb for 2–3 min, followed by washing with 50 μl of 50 mM $NH_4HCO_3$ and spin-drying (20). Spectra were obtained on a time-of-flight spectrometer (Bio-Ion/Applied Biosystems 20 K, Uppsala, Sweden) at an accelerating voltage of 15 kV. ESMS was performed with a Vestec (Houston) 201A quadrapole instrument fitted with an electrospray interface, by infusion of solutions of HEL derivatives in methanol/1% acetic acid, 1:1 (vol/vol) at ≈1 pmol/μl.

**Tryptic Peptide Mapping Analysis *in Situ*.** *In situ* peptide mapping analysis on the NC target surface was essentially performed as described (21, 22). Disulfide bridges of adsorbed HEL derivatives (15 μg) were cleaved at 20°C by addition of 5 μl of dithiothreitol at 10 mg/ml in 50 mM $NH_4HCO_3$. After 20 min the reduced protein was spin-dried, and reagents were removed by washing with 5–50 μl of 50 mM $NH_4HCO_3$. Proteolytic digestion was subsequently performed for 30 min at 37°C under a microscope coverslip by addition of 5 μl of L-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma; 1 μg/μl) in 50 mM $NH_4HCO_3$ (pH 8.3). Samples were washed with 5 μl of 0.1% trifluoroacetic acid prior to PDMS analysis.

**Structure Analysis of HEL.** The structure of HEL (file 7LYZ of the Brookhaven Laboratory Protein Data Bank) was examined on a PS330 interactive display (Evans & Sutherland) by using the PSFRODO (23) version of FRODO (24).

## RESULTS AND DISCUSSION

**Molecular Weight Determination of Ac-HEL and DHCH-HEL Derivatives.** Acetylation reactions of HEL were carried out at pH 7 with up to 10,000-fold molar excess acetic anhydride, and the extent of acetylation was determined by direct PDMS analysis. PD spectra of all Ac-HEL derivatives showed a distribution of singly and multiply protonated molecular ions similar to that of unmodified HEL—e.g., for a HEL sample acetylated at a 1600-fold excess of anhydride yielded an average mass increase of 198 atomic mass units, corresponding to introduction of 5 acetyl groups (Fig. 1). A gradual molecular weight increase at increasing reagent concentration was found (Fig. 2*a*). A maximum uptake of 11 acetyl groups, indicating additional acylation beyond the seven amino groups (six lysine residues and the N terminus), was observed at 10,000-fold reagent excess.

Modification reactions at the arginine-guanidino function have previously been studied with various bifunctional aldehydes and ketones, which in most cases yield heterogeneous products (25). In contrast, the selective formation of DHCH-modified proteins is obtained with CHD (15–17). HEL derivatives obtained with CHD under mild conditions (sodium borate buffer, pH 8.5–9) were analyzed by PDMS at various reaction times (Fig. 2*b*) and showed a molecular weight increase (112 atomic mass units per DHCH residue) due to rapid modification of two arginine residues and a maximum uptake of four DHCH groups. Under the reaction conditions used, no further modification of the 11 arginine residues in HEL was obtained (see Table 2).

PDMS analysis of all Ac-HEL and DHCH-HEL derivatives yielded molecular ion abundances comparable to native HEL, in contrast to dramatically decreased or undetectable molecular ions in PD spectra of dithiothreitol-reduced and alkylated HEL derivatives. A strong suppression of molecular ion desorption was observed as a characteristic feature in PDMS of larger proteins upon denaturation (K. Schneider, D.S., and M.P., unpublished data). Molecular weight determinations by ESMS yielded multiple protonated molecular

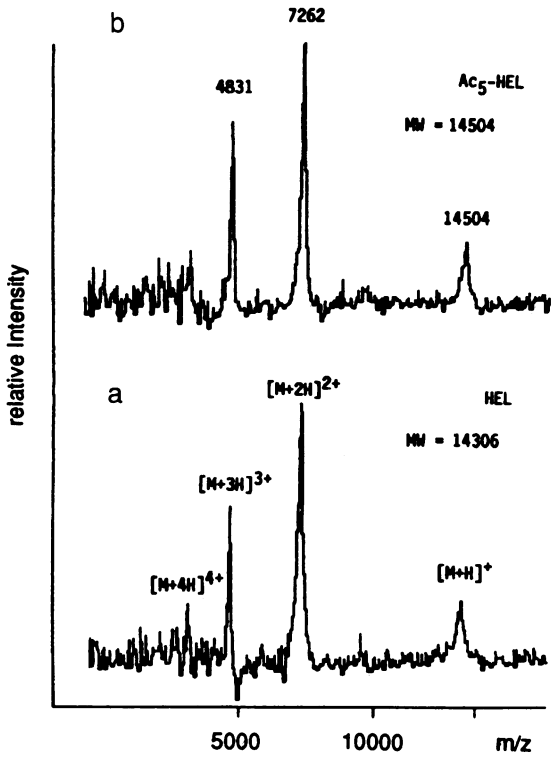


FIG. 1. PD spectra of HEL (*a*) and $Ac_5$-HEL (*b*) obtained with a 1600-fold molar excess of acetic anhydride.

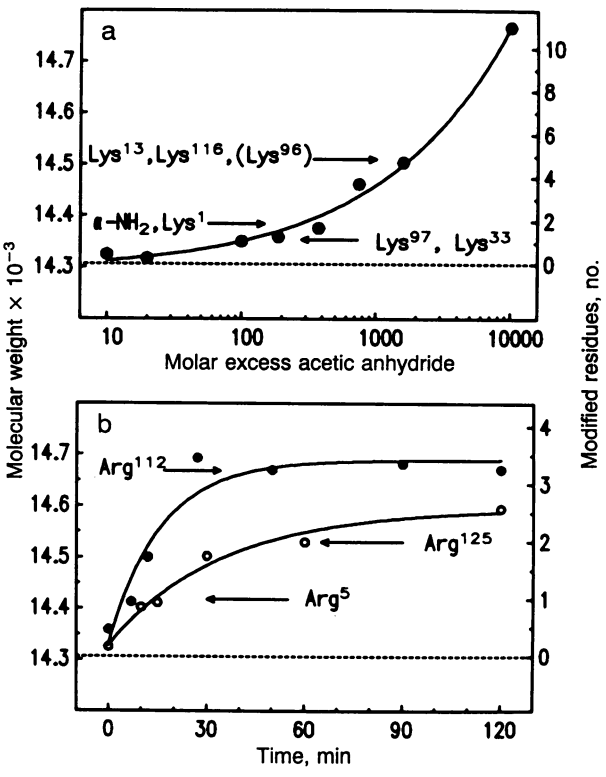


FIG. 2. Molecular weight increase of HEL upon acetylation and DHCH modification. (*a*) Acetylation of HEL with different concentrations of acetic anhydride. (*b*) Reaction of HEL with 50 mM (○) and 150 mM (●) CHD. Modified residues as determined by peptide mapping are assigned by arrows.

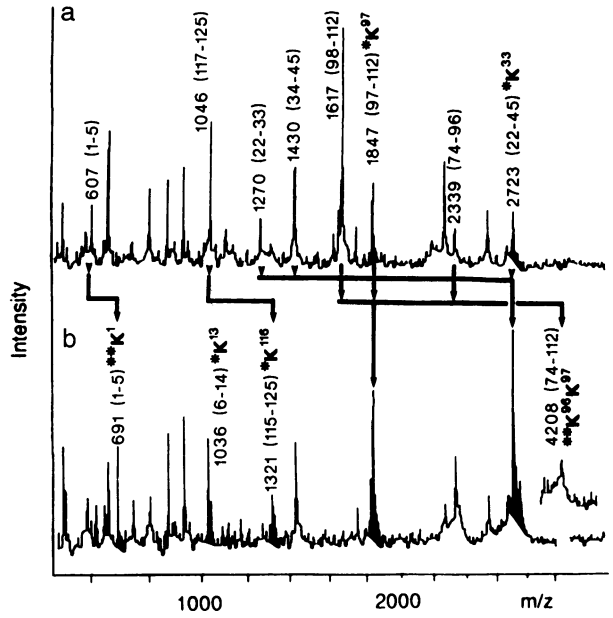


FIG. 3. *In situ* PDMS peptide mapping analysis of monoacetylated ($Ac_1$-HEL) (*a*) and pentaacetylated ($Ac_5$-HEL) (*b*) lysozyme on the NC target surface. Numbers denote $[M + H]^+$ ions and sequences of tryptic peptides—for instance, 607 (1-5) represents HEL-(1–5) pentapeptide of $[M + H]^+$ value 607, and 691 (1-5)** $K^1$ represents [$Ac_2$-$Lys^1$]HEL-(1–5) pentapeptide of $[M + H]^+$ value 691. Acetylated lysine (K) residues are marked by asterisks. Unmodified and corresponding acetylated tryptic peptides are connected by arrows.

ion envelopes of Ac-HEL and DHCH-HEL similar to those of native HEL, indicative of a native-like structure (26), while much more highly charged molecular ions were obtained for unfolded (e.g., alkylated) HEL derivatives (data not shown). Furthermore, no evidence for significant structural changes has previously been detectable by ORD/CD of HEL upon lysine acetylation (27), and complete recovery of enzymatic activity was found upon removal of DHCH groups (17). These results are consistent with a globular tertiary structure maintained in the Ac-HEL and DHCH-HEL derivatives.

**Peptide Mapping Analysis of Reduced and Alkylated Lysozyme.** PDMS peptide mapping analyses of HEL after dithiothreitol reduction and alkylation with 4-vinylpyridine or iodoacetamide were performed by trypsin digestion in solution and PDMS as described (22) and by *in situ* digestion of dithiothreitol-reduced HEL on the NC surface (data not shown). With both methods the complete sets of tryptic peptides of HEL were identified. The *in situ* digestion (21) proved advantageous for the relatively labile DHCH-peptides with regard to sensitivity by obviating sample purification steps and reducing α-chymotrypsin-analogue reactivity of trypsin (28). By contrast, autoproteolytic tryptic background ions were found to be increased (29) but did not interfere with the unambiguous identification of tryptic HEL peptides.

**Correlation of Reactivity and Surface Accessibility of Lysine Residues in HEL.** The acetylated HEL derivatives were subjected to *in situ* peptide mapping analysis as shown in Fig. 3 for two proteins containing one and five acetyl groups, respectively. Molecular ions of all relevant tryptic peptides were found, which enabled the direct identification of acetylation sites (see Table 1). In the spectrum of monoacetylated HEL ($Ac_1$-HEL; Fig. 3*a*), $[M + H]^+$ ions at *m/z* 1847 and 2723 were due to initial acetylation at Lys-97 and Lys-33, which thus can be considered the most reactive residues. Since the peptide fragment [Ac-$Lys^{97}$]HEL-(97–112) is formed by cleavage at Lys-96, acetylation of the latter residue is excluded under these reaction conditions. The partial acetylation of Lys-97 and Lys-33 in $Ac_1$-HEL is evident from the occurrence of the modified peptide ions as well as from the abundant unmodified peptides, HEL-(98–112), HEL-(22–33), and HEL-(34–45). With proceeding acetylation (Fig. 3*b*), ion intensities of the Lys-97- and Lys-33-acylated peptides increased, while ions of the unmodified peptides diminished. At a 400-fold excess of anhydride, 2-fold acetylation at Lys-1 was identified by the ion at *m/z* 691, [$Ac_2$-$Lys^1$]HEL-(1–5), as evident by the exact mass shift from the peptide HEL-(1–5), without observation of an intermediate monoacylated peptide ion. In the further course of the acetylation (1600-fold excess of anhydride), partial modification was also found at Lys-13 and Lys-116 (Fig. 3*b*); under these conditions the final acetylation of Lys-96 was identified by the peptide [Ac-$Lys^{96,97}$]HEL-(74–112) at *m/z* 4208, while ions at *m/z* 2339, HEL-(74–96), and *m/z* 1847, [Ac-$Lys^{97}$]HEL-(97–112), were still abundant. From the consistent, highly reproducible increase of the modified peptide ion abundances with proceeding acylation, concomitant with progressive loss of the unmodified peptide ions (i.e., appearance of modified tryptic arginyl peptides instead of lysyl peptides), relative reactivities of lysine residues could be derived as shown in Table 1, while no exact mass spectrometric quantification of tryptic peptides was aimed at in this study.

To evaluate a correlation with established structural parameters of HEL, relative reactivities of lysine acetylation as determined from the peptide mapping data were compared with the $pK_{1/2}$ values as described by Spassov *et al.* (30) and with SA values for a 1.4-Å van der Waals sphere (18). A clear correlation of chemical reactivities with SA values for the amino groups was found, while ionic effects were not significant (Table 1). The most rapid acylation of Lys-97 and Lys-33 corresponds to the highest accessibilities for these residues (47% and 40%), in contrast to Lys-96 with very low reactivity and the lowest SA value (22%). The relatively rapid 2-fold acylation of Lys-1, in spite of the low accessibility of

Table 1. Comparison of relative reactivities of lysine residues in HEL from PDMS peptide mapping data with surface accessibility and $pK_{1/2}$ values

| Acetylated peptide | *m/z* | Relative reactivity | Residue | SA,* % | $pK_{1/2}$† |
|---|---|---|---|---|---|
| [Ac-$Lys^{97}$]HEL-(97–112) | 1847 | 1 | Lys-97 | 47.4 | 10.5 |
| [Ac-$Lys^{33}$]HEL-(22–45) | 2723 | | Lys-33 | 40.1 | 9.7 |
| [$Ac_2$-$Lys^1$]HEL-(1–5) | 691 | 2 | Lys-1 | 27.9 | 11.2 |
| | | | N terminus | 4.1 | 7.0 |
| [Ac-$Lys^{13}$]HEL-(6–14) | 1036 | 3 | Lys-13 | 26.4 | 11.2 |
| [Ac-$Lys^{116}$]HEL-(115–125) | 1320 | | Lys-116 | 25.8 | 9.9 |
| [Ac-$Lys^{96,97}$]HEL-(74–112) | 4208 | 4 | Lys-96 | 21.9 | 10.9 |

*SA values of amino acids are determined for a 1.4-Å van der Waals radius relative to the tripeptide Ala-Lys-Ala (18).

†pK values of single lysine residues at 0.2 molar ionic strength as described by Spassov *et al.* (30).

Table 2. Relative reactivities of arginine residues and corresponding SA values of $N^7$- and $N^8$-guanidino functions and $pK_{1/2}$ values

| Peptide | *m/z* | Relative reactivity | Residue | SA,* % | $Q_{SA}$† | $pK_{1/2}$ |
|---|---|---|---|---|---|---|
| DHCH-modified | | | | | | |
| [DHCH-Arg$^5$]HEL-(2–14) | 1567 | 1 | Arg-5 | 0/26 | 0.0 | 12.1 |
| [DHCH-Arg$^{125}$]HEL-(117–128) | 1474 | 2 | Arg-125 | 10/24 | 0.42 | 12.3 |
| [DHCH-Arg$^{112}$]HEL-(97–114) | 2187 | 3 | Arg-112 | 16/37 | 0.43 | 12.4 |
| [DHCH-Arg$^{73}$]HEL-(69–96) | — | 4 | Arg-73 | 18/51 | 0.35 | 12.1 |
| Unreacted | | | Arg-14 | 33/37 | 0.89 | 12.4 |
| | | | Arg-21 | 31/34 | 0.91 | 12.6 |
| | | | Arg-45 | 26/30 | 0.87 | 12.5 |
| | | | Arg-61 | 27/41 | 0.66 | 13.4 |
| | | | Arg-68 | 16/25 | 0.64 | 12.6 |
| | | | Arg-114 | 31/33 | 0.94 | 12.1 |
| | | | Arg-128 | 46/51 | 0.90 | 12.0 |

*Relative SA values for $N^7/N^8$ functions of guanidine groups.
†Ratio of SA values for $N^7$- and $N^8$ functions.
‡Estimated by decrease in abundance of tryptic peptide ion *m/z* 2335 HEL-(74–96); the DHCH-modified peptide was not observed.

the N-terminal amino group partially shielded by Thr-40 (27), can be explained by its nucleophilicity ($pK_{1/2}$ = 7.0) and/or a conformational change upon aminoacylation of Lys-1. These results provide evidence that relative reactivity data from mass spectrometric peptide mapping correlate with the accessibility of lysine residues on the protein surface.

**Structure–Reactivity Correlation of the Arginine Modification by CHD.** *In situ* peptide mapping analyses of DHCH-HEL derivatives, performed after different reaction times at 50 and 150 mM CHD, showed a selective modification of Arg-5, -125, -112, and -73, while other arginine residues did not react at a level detectable by PDMS (Table 2). The initial modification was identified at Arg-5 by abundant $[M + H]^+$ ions (*m/z* 1567) of the peptide [DHCH-Arg$^5$]HEL-(2–14) after *ca.* 15 min (50 mM CHD), consistent with the results of Patthy and Smith (17). A high reactivity was also observed for Arg-125 by the abundance of the peptide ion [DHCH-Arg$^{125}$]HEL-(117–128) (*m/z* 1474) concomitant with the diminished unmodified peptide ion HEL-(117–125), while modification of Arg-112 was found in the further course of the

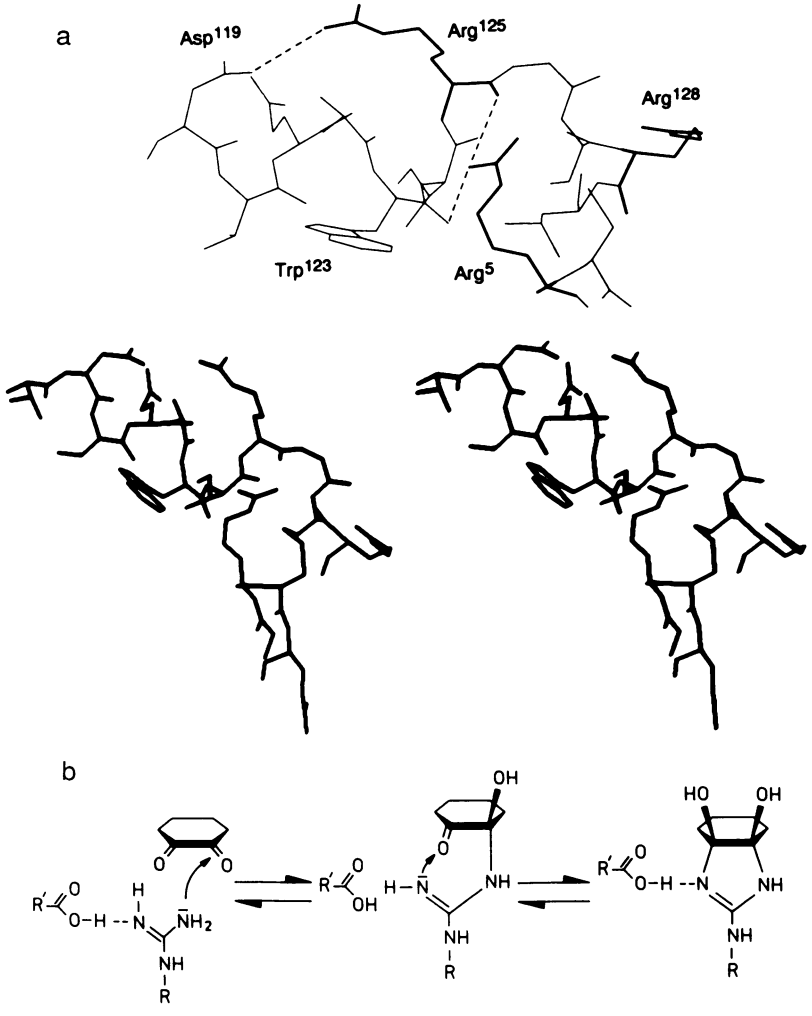


FIG. 4. (*a*) Stereographic presentation of the tertiary structure at Arg-5 and Arg-125 in HEL. Interactions of the $N^7$-guanidine functions of Arg-125 with Asp-119 carboxylate and of Arg-5 with peptide carbonyl groups of Trp-123 and Arg-125 are indicated. (*b*) Reaction mechanism of the DHCH modification assisted by an intramolecular proton acceptor. R, arginyl residue; R', proton acceptor residue.

reaction (60 min with 150 mM CHD) by the ion at $m/z$ 2187, [DHCH-Arg$^{112}$]HEL-(97–114), and the decreasing abundance of the peptide HEL-(98–112). The partial modification of Arg-73 was indicated only by the decreased abundance of the unmodified peptide HEL-(74–96) without direct observation of the corresponding DHCH-modified peptide ion.

The relative reactivities of the arginine residues were compared with their $pK_{1/2}$ values and accessibilities (Table 2) by using separate SA values for the $N^7$- and $N^8$-guanidino functions (18) and the ratio $Q_{SA}$ ($SA_{N7}/SA_{N8}$) as an estimate for the asymmetry of their protein environment. In contrast to the lysine acetylation, an inverse correlation of accessibilities was obtained, the most reactive residues Arg-5 and Arg-125 having the lowest SA values. Moreover, the four reactive arginine residues revealed the largest differences in their $N^7$- and $N^8$ accessibilities ($Q_{SA} < 0.5$), while all unmodified residues have considerably higher $Q_{SA}$ values (0.6–0.9). Examination of the environment of the reactive arginine residues with x-ray structural data of HEL shows the presence of effective intramolecular proton acceptors (Fig. 4*a*). The Asp-119 carboxylate group at a 3.27-Å distance to the $N^7$-function of Arg-125 has been identified recently to form an ionic bond (31). The $N^7$-function of Arg-5 (SA = 0) is tightly surrounded by several residues such as Trp-123 and Arg-125 with peptide carbonyl groups at distances (2.71 and 3.14 Å) suitable to function as hydrogen-bond acceptors. A possibility for hydrogen-bond interaction is also found for Arg-112 to Asn-106 (3.14 Å). No suitable proton acceptor group could be identified for any of the unmodified arginine residues. Thus, without excluding additional contributing factors, these results are consistent with a mechanism of intramolecular catalysis for the DHCH-modification requiring deprotonation of the $N^7$-guanidino function as a first step, providing an accessible $N^8$-amino group with high nucleophilic reactivity (Fig. 4*b*).

## CONCLUSIONS

As shown in recent studies on protein structures and structure–function relationships, mass spectrometric peptide mapping provides information about primary structure, modification sites, and function (10, 14, 32). In this study, peptide mapping was used to probe the surface topology of a protein with intact tertiary structure. At conditions of limited chemical modification, relative reactivities could be assigned to specific lysine and arginine residues; they correlate well with surface accessibilities as established structural parameters and provide information about the protein microenvironment. Furthermore, HEL yields complete sequence data by reproducibly forming abundant proteolytic peptide ions and has a highly stable tertiary structure. Thus, it appears to be well suited as a model protein in chemical modification studies that may affect tertiary structures and reactivities (6, 9).

Although other mass spectrometric methods such as ES and fast atom bombardment have been successfully applied to polypeptide mixtures (10, 13, 33), PDMS provides high sensitivity in the mass range of most proteolytic peptides (*ca.* 500–5,000 atomic mass units); particularly, the possibility of direct *in situ* digestion on the NC surface (21, 34) proved to be advantageous for peptide mapping of proteins that are highly resistant to proteolytic degradation. Furthermore, time-consuming sample purification steps are avoided, thus minimizing possible artefacts with labile protein derivatives.

The structure–reactivity correlations in this study suggest that surface accessibility is of primary importance for highly reactive systems such as lysine and acyl anhydride, whereas neighboring group effects may be dominating for systems of low reactivity. The mass spectrometric approach described here presents broad analytical potential in the evaluation of the selectivity of chemical modification agents (7), the elucidation of reaction pathways, and the analysis of protein surface topology. Although only static accessibilities were used for correlation with reactivities, mass spectrometric peptide mapping may also effectively supplement dynamic methods of surface topology analysis, such as two-dimensional NMR (4, 35), and structure simulation approaches.

We thank Dr. W. Bode and Dr. A. D. Karshikov (Max-Planck-Institute of Biochemistry, Munich), for valuable help with the x-ray structure examination of HEL. Support by the University of Konstanz for a guest professorship for one of us (M.M.) is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, by the Fonds der Chemischen Industrie, and by the University of Konstanz.

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