Protein Unfolding by Peptidylarginine Deiminase

SUBSTRATE SPECIFICITY AND STRUCTURAL RELATIONSHIPS OF THE NATURAL SUBSTRATES TRICHOHYALIN AND FILAGGRIN

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Peptidylarginine deiminases, which are commonly found in mammalian cells, catalyze the deimination of protein-bound arginine residues to citrullines. However, very little is known about their substrate requirements and the significance or consequences of this postsynthetic modification. We have explored this reaction in vitro with two known substrates filagrin and trichohyalin. First, the degree and rate of modification of arginines to citrullines directly correlates with the structural order of the substrate. In filagrin, which has little structural order, the reaction proceeded rapidly to structural order of the substrate. In filagrin, which has little structural order, the reaction proceeded rapidly to structural order of the substrate. In filagrin, which has little structural order, the reaction proceeded rapidly to structural order of the substrate. Third, we show by gel electrophoresis, circular dichroism, and fluorescence spectroscopy that the reaction interferes with organized protein structure; the net formation of 10% citrulline results in protein denaturation. Cyanate modification of the lysines in model α-helix-rich proteins to homocitrullines also results in loss of organized structure. These data suggest that the ureido group on the citrulline formed by the peptidylarginine deiminase enzyme modification functions to unfold proteins due to decrease in net charge, loss of potential ionic bonds, and interference with H bonds.

Peptidylarginine deiminases (PAD)1 (protein-arginine deiminase, protein-L-arginine iminohydrolase, EC 3.5.3.15) are a family of Ca2+-dependent enzymes that catalyze the postsynthetic deamination of protein-bound arginine residues to citrullines (1–7). Biochemical studies have revealed that there are two or possibly three different types of PAD enzymes (8–10). The type 1 enzyme is found in the epidermis and to a lesser extent in the uterus. The type 2 enzyme is expressed in muscle, brain, uterus, spinal cord, pancreas, spleen, salivary gland, stomach, thymus, pituitary, submaxillary gland, etc. Type 3 enzyme is present in hair follicle tissue and in the epidermis. The rat and mouse type 2 (11, 12) and part of the mouse uterus type 1 (13) enzymes have been cloned and sequenced. A recent report indicated that the sequence of the type 3 enzyme is highly homologous to the type 2 enzyme (14).

Little is known about the precise function(s) or target substrates of the PAD enzyme in most tissues. PAD deamination was suggested to modify the action of trypsin-like enzymes (4) and trypsin inhibitors (15, 16), interfere with intermediate filament (IF) assembly (17–19) and play a role in rapid cellular turnover in tissues of secretory activity (9). However, the physiological significance of each of these has not been established.

On the other hand, there are two well documented protein substrates of PAD. One is the filagrin, which is present only in orthokeratinizing epidermis. Filagrin can bundle keratin IF into tight arrays in vitro and in vivo (20) due to ionic interactions of its basic charges with the negatively charged keratin IF (21). Filagrin is largely (22) or partly (19) deaminated in differentiated human stratum corneum cells, rendering it neutral in charge. The second known substrate protein is trichohyalin (THH), which is a major differentiation product of the inner root sheath cells of the hair follicle, and of the medulla in the hair fiber and related structures such as porcupine quills (23–26). THH is also expressed to a lesser extent in interfollicular normal human epidermis, and foreskin epidermis (27, 28). Isolated sheep (25, 29), human, and pig (27, 30) THH are highly insoluble proteins. Secondary structure predictions showed that they are likely to adopt a single-stranded α-helical structure (31). A variety of studies have now established that THH is a target substrate protein for deimination by PAD in hair follicle cells (3, 19, 32–36).

However, the details of the reaction of PAD with filagrin and THH or other substrates remain unclear. Moreover, the function of the PAD modification events in these tissues remains unknown. In this study, we have explored the substrate specificity of the type 2 PAD using as substrates mouse filagrin, human THH, and other model peptides of related structures. Our data reveal certain sequence and substrate structural requirements for the enzyme and, for the first time, the consequences of its action, which provide insights into its function in the skin.

EXPERIMENTAL PROCEDURES

Materials—The following were purchased: type 2 rabbit skeletal muscle PAD (PanVera Corp., Madison WI); sequencing grade Asp-N protease (Boehringer Mannheim); precast Tris-glycine, Tricine, and isolectric focusing (pH 3–7 and 3–10) gels (Novex, San Diego, CA); horse heart myoglobin, protamine sulfate, and a synthetic copolymer polypeptide of average composition alanine-lysine-glutamate-tyro-

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Peptide Hydrolysis—Samples were subjected to acid hydrolysis (5.7 M HCl, 110 °C, 22 h, in vacuo), and their amino acid compositions were determined on a Beckman 6300 amino acid analyzer. The amount of citrulline was corrected for hydrolytic losses to ornithine. Citrulline and ornithine eluted at 15.7 and 50.1 min, near glutamic acid and lysine, respectively. Protein concentrations of samples were determined according to Bradford (37), and by amino acid analysis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemmli (38).

Expression and Purification of Human Trichohyalin Domain 8—The construct of THH-8 (residues 1250–1849 predicted by secondary structure analysis) for expression was made by polymerase chain reaction with primers from the sequences of domain 8 (31), and confirmed by sequencing. The ATG start and TGA stop codons were inserted at the positive and negative polymerase chain reaction primers, respectively. This construct was cloned into the pET-11a vector (Novagen, Madison, WI) (39). The vector was transformed into the BL21 E. coli strain, and bacteria (1–5-liter cultures) were grown in Terrific Broth (Sigma; modified, supplemented with 10 g/liter glucose and 50 g/ml carbenicillin). The bacteria (10,000 g pellet) were equilibrated by dialysis into 10 mM Tris-HCl (pH 8.0) and 1% Triton X-100 several times. The pellet was solubilized in 8 M urea, 20 mM Tris-HCl (pH 8.8), loaded, and chromatographed at a flow rate of 15 ml/h on a Sepharose Q Fast Flow column equilibrated in this buffer. The column was then washed in the same buffer without urea, and the THH-8 was eluted with a linear gradient of 0–1.0 M NaCl. THH-8 was identified by Western blotting with poly- and monoclonal anti-pig THH antibodies (27). The purity and concentrations of the combined fractions was determined by amino acid analysis.

Modifications of Proteins and Peptides with PAD—THH-8 (50 μg/ml) was incubated with PAD at 37 °C at different enzyme-substrate molar ratios (between 1:1000 and 10:1) in a buffer of 20 mM Tris- HCl (pH 8.8), 0.3 M NaCl, 1 mM EDTA, 10 mM dithiothreitol, 5 mM CaCl₂, and the reaction was terminated as required by the addition of EDTA (10 μM final concentration). To determine the extent of reaction (i.e. appearance of citrulline), samples obtained at low enzyme-substrate ratios were acid-hydrolyzed directly for amino acid analysis. Control experiments showed that the PAD enzyme alone does not generate citrulline during reaction. When high enzyme-substrate ratios were used, the reaction products were first resolved on 10% Tricine gels (Novex) by SDS-PAGE, and either pH 3–7 or 3–10 isoelectric focusing gels. The reaction products were then reacted with potassium cyanate for up to 24 h to convert lysine residues to homocitrullines (2). After reaction, the protein samples were electrophoresed by dialysis into 10 mM Tris-HCl (pH 7.5). The extent of reaction was followed by acid hydrolysis, although 35% of the homocitrulline was converted back to lysine under the conditions used (42). Homocitrulline eluted from the Analyser column at 26.0 min.

Physicochemical Measurements—THH-8 (0.05–0.1 mg/ml), mouse filaggrin (0.2 mg/ml), myoglobin (0.5 mg/ml), or copolymer polypeptide (0.35 mg/ml) solutions were equilibrated into the same buffer as used for the PAD reaction. Steady state fluorescence excitation and emission spectra were recorded on a photon-counting spectrophluorometer (Fluoromax Instruments, Paris, France). The bandwidths of excitation and emission monochromators were in the range of 2–4 nm. In all fluorescence experiments, spectra were corrected for Raman contributions by buffer base-line subtraction. Absorption and circular dichroism (CD) measurements were carried out using a Jasco Uvidec 650 spectrophotometer and Jasco 600 and 700 spectropolarimeters, respectively. In both cases, 0.1-cm quartz cuvettes were used. The sample holders were thermostated before and during measurements, using external circulation. Protein concentrations were measured by amino acid analyses to calculate molar ellipticity values for estimation of helix contents. In addition, α-helical contents were routinely estimated by use of curve-fitting algorithms from the shape of the spectra, especially in the case of the copolymer polypeptide of heterogeneous molecular weight. The two methods yielded identical estimates.

RESULTS AND DISCUSSION

To date, studies with the PAD enzyme have utilized mostly model peptide substrates. The purpose of the present study was to explore the specificity and structural requirements of the PAD enzyme using the known in vivo substrate proteins mouse filaggrin and a more soluble cloned portion of human THH (THH-8). For the enzyme, we have used the commercially available type 2 enzyme from rabbit skeletal muscle. While there are minor differences in the antigenicity and apparent molecular weights of the type 2 (“ubiquitous”) and 3 (“hair follicle”) enzymes, they have been reported to have essentially identical substrate specificities (8, 9, 14).

PAD Modification of Mouse Filaggrin—Mouse filaggrin (30 kDa) contains 16% arginine (40 residues/mol) (43). By amino acid analysis, an average of 95% were deiminated to citrullines within 3 h using an enzyme-substrate ratio of 1:1000 (Fig. 1 A), so that the total citrulline content was >15%. To determine whether the 95% conversion meant that all arginines were equally modified or whether ~2 were not modified at all, the filaggrin sample was digested with Asp-N protease, and the 15 fragments encompassing the entire sequence were separated by reverse-phase HPLC. The peptides were subjected to acid hydrolysis, and their amino acid compositions were determined. In all fragments ~95% of the arginine was modified. In confirmation, one fragment containing 4 arginines (Table I) was directly sequenced by Edman degradation. At each expected arginine residue position, >95% citrulline was recovered. The partially and fully modified filaggrin migrated considerably more slowly by SDS-gel electrophoresis (Fig. 1 B).

Expression and Purification of Human THH-8—Although THH can be easily purified from hair follicle tissue (25) or pig tongue epithelium (30) because of its extraordinary insolubility, this precludes its use in in vitro enzymatic reactions. Instead, we have expressed in bacteria domain 8, the largest domain representing about 30% of human THH. This domain consists of numerous irregular peptide repeats of about 26 residues that are typical of the entire human THH sequence (3). The 26-kDa protein can be expressed in E. coli and purified to homogeneity (Fig. 2, inset, lane 1). Its amino acid composition is as expected from its known sequence (including 137 arginines, 22.8%), and is ~86% α-helical as determined by CD...
Fig. 1. PAD deamination of mouse filaggrin. A, conversion rate of arginines to citrullines using a PAD:filaggrin ratio of 1:1000. The data are the averages of three experiments. B, SDS-PAGE for 0, 10, 20, 40, 60, 120, 180, and 1080 min, respectively.

(see Fig. 5B, line 1). Since this result is exactly as predicted for this THH sequence and is consistent with the available data for native pig tongue THH (31), the data indicate that the bacteria expressed protein had properly adopted the native structural configuration. The THH-8 cross-reacts with existing monoclonal (Fig. 2, inset, lane 2) and polyclonal (data not shown) antibodies (27). In the pH 8.8 buffer, its solubility was limited to ~100 µg/ml.

PAD Modification of Human THH-8—The conversion of arginines to citrullines with time as a function of enzyme concentration was assayed by amino acid analysis and was markedly slower in THH-8 than for filaggrin. Using the low enzyme:substrate ratios used with filaggrin (1:1000), very little modification occurred (Fig. 3A, line 1). At ratios of 1:100–1:50, about 20–25% of the arginine content was modified (line 2), but a maximum of up to about 65% could be achieved at higher ratios (up to 10:1) (line 3). As was found for filaggrin, the migration of the modified THH-8 on SDS gels was reduced in a gradual fashion so that its $M_{app}$ was about 160 kDa (Fig. 3B). By isoelectric focusing, bands of progressively more acidic charge were seen (Fig. 3C), from pI ~6.5 for unmodified THH-8, which only enters the gel poorly, to pI 4.8, commensurate with the expected loss of arginine. We also noted that partially to maximally modified THH-8 samples became more soluble (>0.5 mg/ml).

We next wanted to determine why the modification of THH-8 proceeded to only about 65%. Aliquots of unmodified and maximally modified THH-8 were digested with Asp-N, and the fragments were separated on reverse phase HPLC. In the latter, the elution times of the peaks were largely unchanged, probably because most peptides were relatively long (>20 residues), but the peaks became more broad (data not shown). This may be because each peak constituted a mixture of forms of a single peptide whose several arginines were modified to varying extents. By amino acid analyses, most peptides showed 30–70% modification. Many peptides were then partially or completely sequenced, encompassing about 40% of the THH-8 protein (see Table I for partial list). Due to the highly repetitive nature of THH-8, these are representative of >80% of the protein. Indeed, we found that the citrulline contents varied across the broad peptide peaks. However, in general, most of the arginines were converted to citrulline by >75%, except those arginines followed by a glutamic acid residue, which were only slightly modified at ≤5%. Since about one-third of the arginines are followed by glutamic acid residues, this provides a simple explanation for the observed maximal extent of modification. In the special cases of two (Table I, peptides 2, 5, and 7) or three (peptide 1) consecutive arginines in THH-8, the first was completely but the second and/or third were modified less efficiently. In addition, intact modified THH-8 was sequenced from its amino terminus (Table I, peptide 1) to show that arginines followed by aspartic acid residues were modified to near completion.

Progressive Decreases in The Mobilities of Filaggrin and THH-8 after PAD Modification May Be Due to Alterations of Their Conformation and Charge—Changes in mobilities of proteins in SDS fields are often due to changes in conformation and/or charge (44). To further explore this issue, we included 8 M urea in the SDS gel and the sample buffer prior to boiling. In both cases, the unmodified as well as partially modified filaggrin (Fig. 4A) and THH-8 (Fig. 4B) samples all migrated at the decreased rates of ~60 or ~160 kDa, respectively, close to the modified forms. Both of these proteins have unusual structures. Filaggrin is thought to possess a β-turn secondary structure (21) so that the molecule adopts a compact form, which would be expected to be unfolded by urea into a larger molecular volume. THH-8, like intact THH itself, is thought to form a single-stranded α-helix in which successive turns of the helix are stabilized by intrachain ionic bonds (31, 45) to form an elongated rodlike shape, which might not be completely unfolded by SDS. In these cases, urea may be a more efficient protein denaturant than SDS. Indeed, numerous other reports have documented that SDS denaturates some proteins incompletely (44, 46, 47). When arginine is converted to citrulline, the guanidino group is replaced by a ureido group. This modification would be expected to destroy many potential salt bonds and interfere with H bonds, which could thus destabilize the protein structures, resulting in unfolding. Thus we speculate that the progressive mobility decreases caused by partial PAD modification may be related to progressive unfolding, i.e. maximal modification of filaggrin and THH-8 by PAD may result in changes in structure that are comparable with denaturation by urea.

In addition, the mobility changes may be due to changes in charge of the target protein. This is especially true of arginine-rich basic proteins, and proteins rendered neutral or acidic in charge following chemical modification (48–50). For example, the mutation of a single arginine residue resulted in significantly decreased mobility of a protein (51). It is possible that the net gain of many more acidic charges on the proteins arising from loss of arginines may in effect explode the proteins due to adverse charge interactions. Consistent with this, we could modify 75% of the arginines of protamine sulfate to generate a citrulline content of ~50%. In this case, the protein became neutral in charge, soluble in SDS, and migrated with an apparent molecular weight twice its expected size (data not shown). Mobility changes were also seen with histones (final citrulline contents of up to ~10%).

Thus in the cases of the arginine-rich proteins THH-8 and filaggrin, the unusual mobility changes induced by PAD mod-
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Extents of modification of arginines in synthetic peptides and peptides derived from proteins

The numbers above the arginine residues show the approximate percentage converted to citrulline. For THH-8 peptides, the sequences shown are representative of >80% of the total protein. In this case, most peptides were not sequenced to completion; the lowercase letters define the remainder of the sequence of the long peptide, as ascertained by amino acid analysis. Peptide 1 was sequenced from intact THH-8.

| Origin (Ref.)       | Sequence                   | Secondary/Structure       |
|-------------------|----------------------------|---------------------------|
| Peptide from mouse filaggrin | DSQVHSGQVQVEGRRQGGSSANARAA 5 | 908090 0 0 90              |
| Peptides from human THH-8 | 1. PEEEQLEREEQKEAKRDRK5QEEQLLREEREEXK 8070 9090 | RRQETDROK5QEEKQLL... 85 5 9560 50 0 2. DRKFREEQEQVQERERKFLEEQQlqrrhrrkfr eeqllgrereeqlqrrq | 80 0 95 70                       |
|                   | 3. DRKFREEQEQVQERERKFLEEQQlqrrhrrkfr eeqllgrereeqlqrrqfgq | 85 0 95 0 4. DRKFREEQEQVQERERKFLEEQQlqrrq | 9585                          |
|                   | 5. DEQLLQEREEQQqlrrqer 90 | 9575 0                        |
|                   | 6. DEQLLQEREEQQqlrrqer 90 | 9575 0                        |
|                   | 7. DRKFLEEQQDLRRQEQRLQRQlrrqer 85 0 | 9585                        |
|                   | 8. DRKFREEEQLLQEGEEEQqlrrqer 95 | 9585                        |
|                   | 9. DGRYRREEEQLLQEEQQlqleeqqlqrrqer 80 95 | 9585                        |
|                   | 10. DRQYRAEEQFATQERKsrreeqelqweeqqkrrqere rklreehirrqqkeeqhr 95 95 95 95 | Random coil (β-turn motifs); unchanged |

Synthetic peptides

Mimetic filaggrin (Mack et al., 1995) SGSRSGRSGSRSGRSGS Random coil (β-turn motifs); unchanged

Human keratin 1 subdomain 1A (Chipev et al., 1993) REQIKSLNNGFAFSDK α-Helical; unchanged

Human keratin 10 1A subdomain (Zhou et al., 1988) GRVTMQLNDRLASYLDRVRALEESNYELEGKIKERYD α-Helical to disordered structure

Human THH (Lee et al., 1993) RQERDKFREEEQQRLRQEEREQQQLR α-Helical to disordered structure

Mimetic THH 80 9595 70 GQERDKFREEEQ 0 100907050 100859595 REQQLRRQEREREQQQLRQERDRKF Random coil; unchanged α-Helical to disordered structure

The numbers above the arginine residues show the approximate percentage converted to citrulline. For THH-8 peptides, the sequences shown are representative of >80% of the total protein. In this case, most peptides were not sequenced to completion; the lowercase letters define the remainder of the sequence of the long peptide, as ascertained by amino acid analysis. Peptide 1 was sequenced from intact THH-8.

Biophysical Measurements Confirm That PAD Modification Reduces the Degree of Structural Order of Proteins—The postulated structural consequences of the PAD reaction were examined directly using biophysical analyses. As expected (21), by CD, filaggrin is mostly α-helical (Fig. 5B, line 1), as predicted from its sequence, and it is slightly more ordered than observed for intact native pig tongue THH (31). Conversion of 25% (line 2), 40% (line 3), or 65% (line 4) of its arginines, generating citrulline contents of ~5.5%, 9%, and 15%, reduced its α-helical contents to ~60%, 35%, and to no organized structure, respectively. This loss of α-helicity observed in lines 3 and 4 is comparable with denaturation of the THH-8 by 2.5 M or 4.5 M urea, respectively (Fig. 5C).

We also used fluorescence spectroscopy. The spectrum of THH-8 upon excitation at 280 nm (to visualize both tyrosines and tryptophans) is blue-shifted, and the peak at 312 nm suggests that the two tryptophan residues are buried within the protein structure (Fig. 6A, solid line). However, following maximal modification by PAD, the red shift in the fluorescence spectrum toward longer wavelengths (dotted line) is similar to that of THH-8 fully denatured in 6 M urea (dashed line). These data indicate that upon maximal modification by PAD, the two tryptophans have become exposed and thus freely mobile, which is consistent with the concept of loss of organized structure.

Furthermore, steady state fluorescence anisotropy measurements were made (Fig. 6B). On addition of increasing amounts of PAD enzyme to the cuvette containing the THH-8 solution, the anisotropy decreased from 0.21 (unmodified THH-8) to less than 0.15 in protein in which ~40% of the arginines had been modified (~11% citrulline content). This decrease clearly indicates the absence of protein dimerization, as also suggested by the SDS-PAGE data (Figs. 1 and 3), and confirms that the protein had become unfolded by the PAD modification reaction.

Introduction of Homocitrullines Also Interferes with Protein Structures—The foregoing data suggest that the modification of arginines to citrullines results in a progressive unfolding of the proteins equivalent to that obtained with ~4.5 M urea. One possible explanation for this phenomenon is that the substi-
tuated urea side chain of citrulline interferes with ionic and H-bonding interactions. To test this hypothesis, we inserted homocitrulline into proteins by chemical conversion of the ε-NH₂ groupsoftheirlysinesidechainswithcyanate. We used three proteins of widely differing lysine contents but which have highly ordered structures: THH-8 (5.5% lysine, 86% α-helix), intact myoglobin (12.7% lysine, 70% α-helix), and a commercially available random copolymer polypeptide (35.7% lysine, 90% α-helix). Reaction with cyanate for up to 24 h resulted in high degrees of conversion of lysines to homocitrullines and retention of their high solubilities, based on amino acid analyses (data not shown). By CD, the α-helical content of THH-8 was estimated to have been reduced from 86% to 60% (1.6% homocitrulline), 20% (3% homocitrulline), and to 10% (4% homocitrulline) (Fig. 7, A, lines 1–4, respectively). In the case of the lysine-rich synthetic copolymer peptide, the α-helical content was reduced from 90% to 40% (5% homocitrulline), 10% (17% homocitrulline), and to no structure (21% homocitrulline) (Fig. 7B). Likewise, with myoglobin, the α-helical content was reduced from ~70% to 60% (2% homocitrulline), ~15% (7% homocitrulline), and to <10% (10% homocitrulline) (Fig. 7C). In this case, the loss of structure with retained heme group and disulfide bonds was similar to that seen upon denaturation in acid solutions (53, 55, 56).

These data show that the insertion of homocitrulline even in low amounts begins to reduce the high α-helical contents of these proteins. Taken together, the degree of loss of structure correlates with the degree of modification (Fig. 7D). Indeed, comparison of the THH-8 data suggests that homocitrulline seems to be even more effective in unfolding of protein structure than citrulline. Therefore, these data support the concept that the ureido group of the citrulline or homocitrulline side chains promotes the observed interference in organized protein structure.

**Substrate Structural Requirements for PAD Modification**—While some information on the specificity of the PAD enzyme reaction has become available using arginine derivatives or short peptides (57), other data have been generated using model protein substrates, probably unrelated to in vivo substrates. For example, in soybean trypsin inhibitor, one arginine in its active site, located on an external loop, could be modified very rapidly (enzyme:substrate ratio of 1:50 in 10 min) (15), while nine others could be converted more slowly within 5 h (57). However, in mouse plasma trypsin inhibitor, which is largely α-helical in structure, the reaction proceeds at a rate one-tenth that for soybean trypsin inhibitor (15, 16). On the
other hand deimination of IF protein chains occurs only in the non-α-helical head and tail domains (18). Together with the present data on the natural substrates filaggrin and THH-8 (Figs. 1 and 3; Table I), these observations suggested that the PAD reaction may be dependent on substrate structure.

To explore this issue further, we examined the structural requirements of the PAD enzyme reaction with synthetic peptides of filaggrin, THH, and keratins 1 and 10 (Table I). Following deimination at an enzyme:arginine ratio of 1:40,000, the peptides were purified by HPLC, analyzed to determine their citrulline contents, and sequenced (Table I). In most cases the peptide peaks from the HPLC column became broader, suggesting a heterogeneous population of molecules with varying degrees of arginine modification. The mimetic filaggrin peptide of β-turn structure was 85% deiminated, as for the same sequence in intact mouse filaggrin. In the keratin 10 1A peptide of α-helical structure, an average of 70% of the arginines were modified, of which the second arginine from the amino terminus and the arginine third from the carboxyl terminus were converted ~50%, while the two internal arginines were modified 85%, and the α-helical structure of the peptide was lost. Similarly, in the shorter α-helical keratin 1 1A peptide, the amino-terminal arginine was only slightly modified. By CD the longer natural and mimetic THH peptides were >90% α-helical, and thus are more closely related to the structure of THH-8. While their α-helical structures were lost, the degrees of modification of the arginines varied widely. The amino-terminal arginine was not modified at all; most single arginines were completely modified; a single arginine followed by glutamic acid residue was significantly modified to 60–80%, to a much higher degree than in THH-8 or the two longer synthetic peptides.

Altogether, the present data accumulated from mouse filaggrin, human THH-8, and synthetic mimetic peptides show that arginines located near the amino terminus are poorly modified, arginines in highly α-helical protein structures are only slowly modified to near completion, and arginines in proteins of little structural order are rapidly modified to near completion. However, a single arginine followed by a glutamic acid residue is only poorly modified in an α-helical protein, but significantly modified in proteins of low structural order; in the rare cases of two consecutive arginines, the first was always modified to a greater extent than the second arginine. Thus the PAD reaction is dependent on both substrate structure and precise sequence around the arginine residues.

**Concluding Remarks**—The present studies have identified several important properties of the PAD enzyme. First, in
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In general, proteins such as filaggrin having only a simple β-turn secondary structure were quickly and quantitatively modified, whereas those with high α-helical contents were modified more slowly. Second, certain sequence preferences were identified. Thus the activity of the PAD enzyme is markedly affected by both substrate structure and substrate sequence in the vicinity of the arginines. Third, of likely significant biological importance, the present data show that the structural properties of the natural substrates THH and filaggrin are changed on extensive PAD modification. Modification of sufficient arginines to homocitrullines may disorder organized protein structures, and a net content of ~10% causes loss of structure (Figs. 5 and 6). This may be attributable to the ureido group of the citrulline, since a charged electron donor group is modified to an electron acceptor group. This would be expected to interfere with the stabilization of H bonds and is no longer able to form ionic bonds. Chemical modification of lysines to homocitrullines may disorder organized protein structures in the same way (Fig. 7). The unfolding of filaggrin and THH by PAD in the skin would be expected to significantly alter their interactions with IF and transglutaminases (22, 26, 36, 58, 59), questions for which additional experiments will now be required.

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Fig. 7. Insertion of homocitrullines also denatures proteins. CD spectra were taken of THH-8 (A), a synthetic copolymer polypeptide (B), and myoglobin (C) after reaction with cyanate to convert up to 90% of lysines to homocitrullines. The numbers refer to the degrees of cyanate modification, as indicated under “Results and Discussion.” In B the α-helical contents were estimated by computer analyses of the line shapes since the polypeptide is heterogeneous in molecular weight. In D, the estimated α-helical contents of the three proteins were plotted versus homocitrulline content (closed symbols). The data for THH-8 and citrulline (from Fig. 5B) are shown for comparison (open symbols).
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