Deimination of Myelin Basic Protein. 1. Effect of Deimination of Arginyl Residues of Myelin Basic Protein on Its Structure and Susceptibility to Digestion by Cathepsin D†

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ABSTRACT: The effect of deimination of arginyl residues in bovine myelin basic protein (MBP) on its susceptibility to digestion by cathepsin D has been studied. Using bovine component 1 (C-1) of MBP, the most unmodified of the components with all 18 arginyl residues intact, we have generated a number of citrullinated forms by treatment of the protein with purified peptidylarginine deiminase (PAD) in vitro. We obtained species containing 0−9.9 mol of citrulline/mol of MBP. These various species were digested with cathepsin D, a metalloproteinase which cleaves proteins at Phe−Phe linkages. The rate of digestion compared to component 1 was only slightly affected when 2.7 or 3.8 mol of citrulline/mol of MBP was present. With 7.0 mol of citrulline/mol of MBP, a large increase in the rate of digestion occurred. No further increase was observed with 9.9 mol of citrulline/mol of MBP. The immunodominant peptide 43−88 (bovine sequence) was released slowly when 2.7 and 3.8 mol of citrulline/mol of MBP was present, but it was released rapidly when 7.0 mol of citrulline/mol of MBP was present. The dramatic change in digestion with 7.0 mol of citrulline/mol of MBP or more could be explained by a change in three-dimensional structure. Molecular dynamics simulation showed that increasing the number of citrullinyl residues above 7 mol/mol of MBP generated a more open structure, consistent with experimental observations in the literature. We conclude that PAD, which deiminates arginyl residues in proteins, decreases both the charge and compact structure of MBP. This structural change allows better access of the Phe−Phe linkages to cathepsin D. This scheme represents an effective way of generating the immunodominant peptide which sensitizes T-cells for the autoimmune response in demyelinating disease.

Citrulline, an amino acid found uncommonly in proteins, is generated by the deimination of arginyl residues by the enzyme peptidylarginine deiminase (PAD, EC 3.5.3.15). The result of the deimination is conversion of a positively charged arginine to an uncharged citrulline, with the accompanying loss of one positive charge from the protein for each arginine deiminated. Loss of positive charge decreases the ability of the protein to interact electrostatically with negatively charged groups, particularly phosphate groups of nucleic acids and phospholipids. In addition, the conversion of arginine to citrulline has been shown to affect the secondary structure of other structural proteins and the activity of enzymes. Deimination of arginyl residues of trichohyalin resulted in loss of organized structure (*1*), and deimination decreased the ability of the protein to aggregate keratins (*2*). Deimination of arginine 16 in glycogen phosphorylase decreased phosphorylation (by phosphorylase kinase) at serine 14, thereby reducing its activity (*3*). In a decapeptide corresponding to residues 9−18 of glycogen phosphorylase, the presence of citrulline at positions 10 and 16 decreased the activity of protein phosphatase 1 on the phosphorylated peptide (*4*). These data demonstrated that deimination of arginyl residues has important consequences for the structure and function of proteins.

In recent years the deimination of arginyl residues has been implicated as an important reaction in the chemical pathogenesis of two autoimmune diseases, multiple sclerosis (MS)[1] and rheumatoid arthritis (RA). Myelin basic protein (MBP) is an important autoantigen in MS. The 18.5 kDa isoform, the major isoform in the human, has been shown to consist of a number of components ("charge isomers") generated by one or a combination of posttranslational modifications, including deamidation, phosphorylation, methionine sulfoxide, and deimination. The deimination of arginyl residues is of particular interest to us, since an 18.5 kDa charge isomer containing 6 citrullinyl residues (i.e., 6/19 arginyl residues deiminated) has been been found to be increased in victims of demyelinating disease (*5*). In normal human adults this citrullinated charge isomer accounts for 20% of the total. In victims of MS, the proportion of this citrullinated MBP was increased to 45% of the total MBP. In other neurological diseases, e.g., in Alzheimer's, Parkin-

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[1] Abbreviations: MBP, myelin basic protein; component 1 (C-1), MBP−Cit_0 in which all 19 arginyl residues are intact; MBP−Cit_{1-19}, MBP containing 1−19 citrulline residues; MS, multiple sclerosis; charge isomer, MBP species which vary in charge due to posttranslational modifications (these are not isomers in the mass spectrometric sense).

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son's, Huntington's, motor neuron, and amyotropic lateral sclerosis (*5*), the proportion of this charge isomer remained at 20%. In a case of fulminating MS (Marburg's variant), more than 80% of the total MBP was of the citrullinated variety, and instead of 6 citrullinyl residues/mol of MBP,[2] the Marburg's variant contained 18 citrullinyl residues/mol of MBP (*6*). Only a single arginine remained. These data suggested that the severity of the disease correlated with the extent of deimination of arginyl residues in MBP.

In a recent publication, we reported that the presence of 6 citrullinyl residues in human MBP increased its rate of digestion by cathepsin D (a myelin-associated protease) 4-fold, but the presence of 18 citrullinyl residues (Marburg's variant) increased the rate of digestion 35-fold (*7*). In a study of the three-dimensional structures of these MBP molecules by both scanning transmission and transmission electron microscopy, protein−lipid complexes formed by Marburg's variant were determined to be much less compact (*8*), consistent with a less compact structure of the protein, possibly accounting for the increased susceptibility to digestion.

Since cathepsin D has been localized to CNS myelin (*9*) and its activity has been shown to be elevated in MS (*10*), particularly surrounding active plaques (*11*), a more detailed study of the structural factors which affect the activity of the enzyme, in particular, the effects of deimination, was undertaken and is described here. We show that the extent of deimination has little effect on the digestion of MBP by cathepsin D until 5−7 mol of arginine/mol of MBP was deiminated. With 7 mol or more of citrulline in MBP, the digestion increased rapidly, suggesting that a structural change in MBP took place and the Phe−Phe linkages at residues 43−44 and 89−90 (human sequence) were more available to the enzyme. Peptide 44−89, generated by cathepsin D cleavage, has been reported to contain the immunodominant epitope and has been found in the cerebrospinal fluid of patients with MS (*12*, *13*). Moreover, its unexplained unusual stability (*7*), and the mechanism and rapidity of release of this peptide from MBP, may have important implications for our understanding of the chemical pathology of demyelinating disease.

EXPERIMENTAL PROCEDURES

Preparation of MBP. MBP was isolated from bovine brain as described by Wood and Moscarello (*14*). Component 1 (C-1) was isolated by CM52 column chromatography at pH 10.6 in 2 M urea as described earlier. MBP C-1 is the most cationic of the components and therefore elutes last from the column with a 2 M sodium chloride gradient. It is also the least modified, posttranslationally, by acylation at the N-terminus and mono- and dimethylation at arginine 106 in the bovine but arginine 107 in the human MBP.

Deimination of MBP with Peptidylarginine Deiminase. Peptidylarginine deiminase (PAD) was isolated from bovine brain as described earlier (*15*). MBP was incubated with PAD at 52 °C in HEPES buffer, pH 7.6, containing 5 mM calcium chloride and 2 mM DTT, for various times from 0 to 24 h. The reaction was halted by boiling for 5 min and centrifuged at 12 000 rpm in a Hettich microcentrifuge for 10 min. The supernatant, which contained the MBP, was desalted by HPLC using a gradient of 60% acetonitrile (solution B) in 0.05% TFA. The gradient began with 0% solution B, which was increased to 30% in 5 min and then to 80% in 30 min. The absorbance at 226 nm was measured, and the material eluting at 25−28 min was collected and lyophilized. An aliquot was taken for amino acid analysis on a Waters Pico Tag system after hydrolysis in 5.7 N HCl in the gas phase. The amount of citrulline/mol of MBP was computed from these data. For convenience, we have adopted the following nomenclature: MBP C-1, which does not contain citrulline, is termed MBP−Cit_0; the other components of MBP with various amounts of citrulline are termed MBP−Cit_2, MBP−Cit_7, etc.

Digestion of MBP with Cathepsin D and Separation of Peptides on HPLC. Bovine brain cathepsin D was purchased from Sigma (St. Louis, MO). Digestions were done at 37 °C in 0.05 M ammonium acetate buffer (pH 3.5). Enzyme-to-substrate ratios were 1:100 mol/mol. Reactions were terminated by boiling for 5 min. The samples were then frozen and lyophilized prior to resuspension in water for peptide separation on HPLC.

MBP−Cit_x was obtained with 0, 2.7, 3.7, 7.0, 8.9, and 9.9 mol of citrulline/mol of MBP. Each sample was digested with cathepsin D. The peptides resulting from the cathepsin D digestion were separated using an LKB C2/C18 column with a gradient of 60% acetonitrile (solution B) in 0.05% TFA. The gradient began at 0% solution B, which was increased linearly to 30% solution B in 5 min and then linearly to 65% solution B in the subsequent 35 min. The peptides were located by absorbance at 226 nm. The peptides were identified by amino acid analyses and mass spectrometry. Prior to mass spectrometry, the peptides were dried and stored in polypropylene tubes at −20 °C.

Mass Spectrometric Analysis of MBP Peptides. The cathepsin D peptides were analyzed in the Q-Tof mass spectrometer, using Mass Lynx Software version 3.2 fitted with a Z-Spray electrospray inlet system (Micromass Canada, Montreal, Canada). Each sample was dissolved in the ionization solution (50−500 μL) consisting of a 1:1 mixture of acetonitrile (anhydrous DNA synthesis grade; Caledon Laboratories, Georgetown, ON, Canada) and milliQ water containing 0.2% acetic acid (v/v) (ACS grade, British Drug Houses). The sample (10 μL) was pumped into the mass spectrometer by the Alliance Separation Module 2190 (Waters, Mississauga, ON, Canada) at 10 μL/min via an HPLC injector (Valco Instruments) fitted with a 10 μL sample loop. Specifically, samples were introduced into the Q-Tof by infusing their solutions at 5 μL/min with a 500 μL Gastight glass syringe (Hamilton Syringes), mounted on a syringe pump (model 11; Harvard Apparatus), which was connected to the inlet via 75 μm (i.d.) fused silica tubing. The instrument was calibrated in the mass range of 600−1500 amu with a 200 nM solution of horse heart myoglobin (Sigma Chemicals, Mississauga, Canada). Flow rates for desolvation and the nebulization nitrogen gases were 25 and 15 L/h, respectively. The capillary voltage was set at 3200−3500 V, and the cone voltage was set at 30−40 V.

Mass spectra were collected in the mass range 400−2000 amu over a period of 1−2 min at a sampling rate of 1.9 s per spectrum with 0.1 min separation between consecutive spectra. A number of spectra over a fixed period of time,

[2] For convenience, we will refer to bMBP without citrulline as bMBP−Cit_0 and bMBP−Cit_x for bMBP with various amounts of citrulline where *x* may be 1−18 (1−19 for human hMBP).

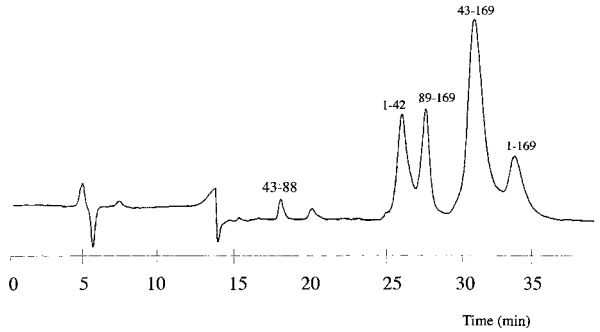

FIGURE 1: HPLC profile of C-1 isolated from bovine MBP (MBP−Cit_0) and digested with cathepsin D (1:100) for 2 h at 37 °C. Four peptides were obtained: (A) 43−88; (B) 89−169; (C) 1−42; (D) 43−169. E (1−169) is undigested MBP−Cit_0.

generally 1 min, were combined and deconvoluted by the maximum entropy program Max Ent (Micromass, Montreal, Canada) in the mass range 15000−25000 amu.

Molecular Dynamics Simulations of Human MBP−Cit_x Models. A three-dimensional atomic structure model of human MBP (hMBP) (Protein Data Bank accession number 1qcl) was used here as a means to test the effects of deimination of specific arginine residues to citrulline residues, using INSIGHT II/97 software (Molecular Simulations Inc., San Diego, CA). The rationale for using the hMBP model was simply that the general effects of deimination would be expected to be the same for all mammalian MBP. Molecular dynamics is a computational means to simulate the movement of atoms and the conformational changes of the protein under specified conditions (here, in a vacuum at 310 K), with a view toward finding a minimal energy form of the molecule, even one lying across a local energy barrier. The process requires the definition of a force field giving the potential energies of atoms; here, ESFF was used. The force field comprises all interactions (electrostatic, hydrogen bonding, van der Waals, and hydrophobic) among atoms in the model.

The 1qcl model was first subjected to energy minimization for 500 iterations by a steepest descent means and then for a further 500 iterations by the conjugate gradient approach. The resulting structure is termed here hMBP−Cit_0 and was the starting point for further simulations. Computational deimination was achieved by deleting the two hydrogens on the terminal amine group and converting the N atom to O. The model hMBP−Cit_6 contained the citrullines usually found in component hMBP/C-8 in chronic MS: residues 25, 31, 122, 130, 159, and 170 (*14*). The model hMBP−Cit_{19} had all 19 arginines converted to citrulline. All three hMBP−Cit_x models underwent molecular dynamics simulations for 5000 iterations in "create velocity" mode, 5000 iterations in "current velocity" mode, and then 500 iterations of energy minimization.

RESULTS

Characterization of Cathepsin D Peptides by Mass Spectrometry. Bovine MBP−Cit_0 was digested for 2 h with cathepsin D at a molar ratio of 1:100 (enzyme/substrate), and the peptides were separated on HPLC (Figure 1). Preliminary identification of the peptides obtained was made by amino acid analyses. Their full identification was made by mass spectrometry (Table 1). The computed and measured masses were in good agreement for all peptides. Peptide 43−88 (bovine sequence) eluted first at 19 min, peptide 89−169 at 28 min, peptide 1−42 at 30 min, and peptide 43−169 at 31−33 min, just ahead of the intact protein (viz., peptide 1−169) at 36−37 min.

Table 1: Calculated and Measured Masses for Peptides Resulting from Digestion of MBP with Cathepsin D

peptide	calculated mass	measured mass
1−169	18365.5	18365.5
1−42 + acetyl	4779.3	4778.9
43−88	4990.4	4989.6
89−169	8631.8	8631.5
43−169	13618.2	13619.9

Digestion of MBP−Cit_x with Cathepsin D following Deimination for Various Times. Deimination of bovine MBP−Cit_0 with PAD for various times generated a number of citrullinated forms, varying in citrulline content from 0 to 9.9 mol of citrulline/mol of protein as determined by amino acid analyses of aliquots of each incubation. These isomers were digested with cathepsin D at 37 °C (1:100, moles of enzyme per mole of protein) for various times from 0 to 24 h, and the peptide products were separated by HPLC. A typical HPLC profile for MBP−$Cit_{3.8}$ digested with cathepsin D is shown in Figure 2. At zero time, the undigested protein MBP−$Cit_{3.8}$ is shown at 33 min. An additional peak eluted at 25 min. Amino acid analysis of the material at 25 min was negative for amino acids, and mass spectrometry failed to yield a spectrum. This material was only found in the deiminated proteins and was never observed in undigested MBP−Cit_0 preparations not incubated with PAD (see Figure 1). When the incubation mixture used for deimination, i.e., without enzyme, was applied to the HPLC, only the intact protein was seen at 36−37 min and nothing was seen at 25 min. At the present time, we do not have an explanation for this material. Since it is not protein, we ignored it in our calculations of peptide concentrations. Similar sets of curves (Figure 2) were obtained for each of the differentially citrullinated species but are not reproduced here.

The effect of deimination on the susceptibility of the intact protein to cathepsin D digestion is shown in Figure 3, in which the percentages of the intact protein remaining after various times of digestion with cathepsin D are shown. With MBP−Cit_0 (Figure 3A), 50% of the intact protein was still present after 30 min of digestion with cathepsin D. This proportion decreased slightly when MBP−$Cit_{2.7}$ and MBP−$Cit_{3.8}$ were used. However, with MBP−$Cit_{7.0}$, the digestion was very rapid. Less than 5% of the initial amount of intact protein remained after 30 min of digestion, suggesting that some change in the structure of the molecule occurred when seven arginines were deiminated. From such curves, the rate of digestion of the intact protein as a function of citrulline content was obtained (Figure 4). Deimination of arginyl residues had little effect on digestion with cathepsin D until MBP−$Cit_{7.0}$ was used, when a rapid increase in rate occurred and remained at this high level for MBP−$Cit_{7.0-9.9}$.

Peptide Bond Phe^{42}−Phe^{43} Was Cleaved First by Cathepsin D. Bovine MBP contains two Phe−Phe linkages, one at 42−43 and the other at 88−89. To determine which linkage was cleaved first, the proportions of peptides 43−169 and 43−88 were plotted as a function of time for MBP−Cit_0, MBP−$Cit_{2.7}$, and MBP−$Cit_{7.0}$ (Figure 5). In panel A, the data for peptide 43−169 are shown, and in panel B, the data

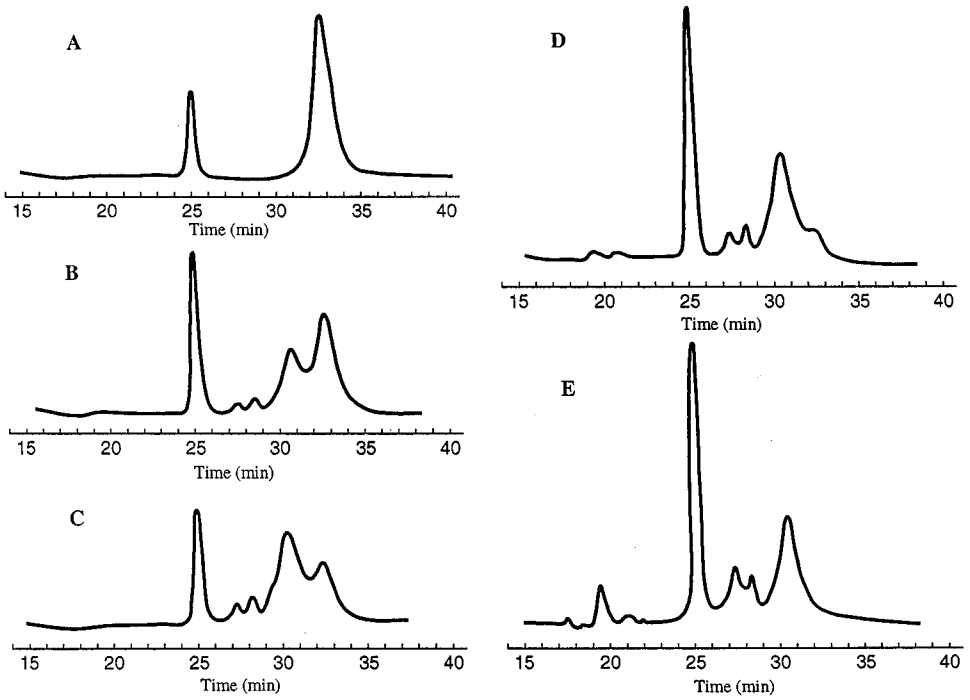

FIGURE 2: HPLC profile of bMBP−$Cit_{3.8}$ digested for different times with cathepsin D: A, 0 min; B, 15 min; C, 30 min; D, 3 h; E, 24 h.

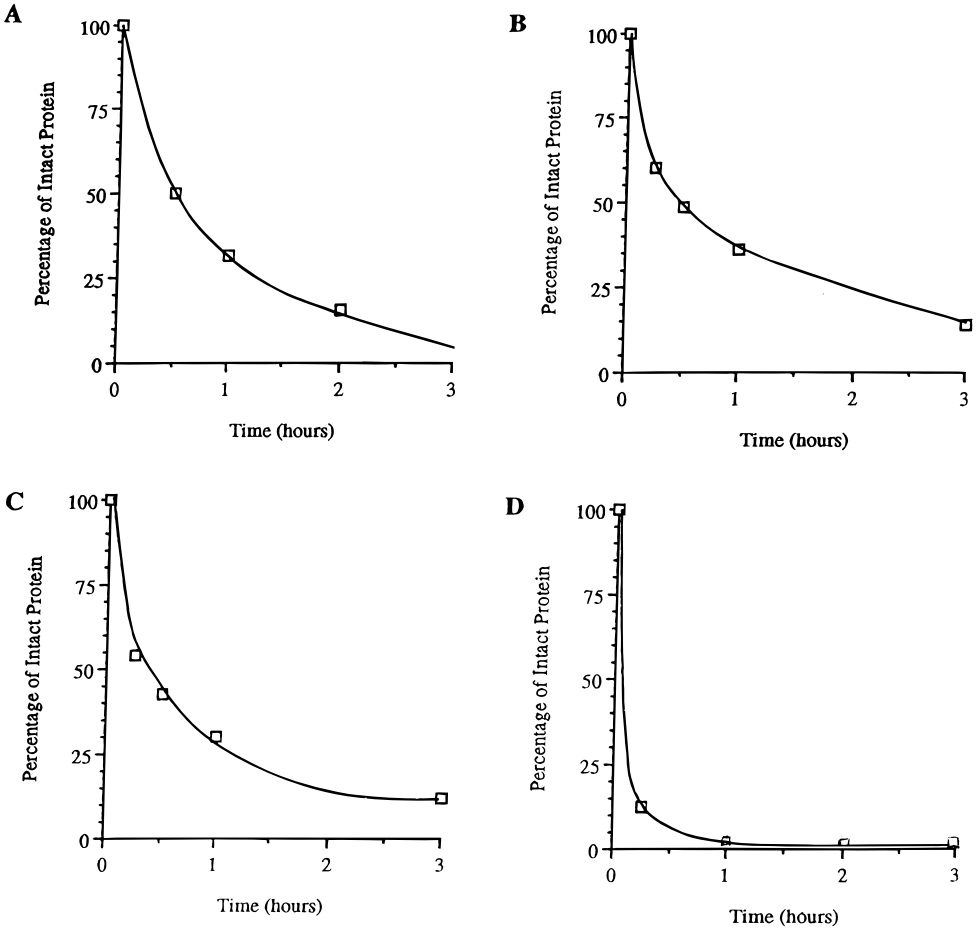

FIGURE 3: Disappearance of intact protein from 0 to 3 h with different numbers of citrullinyl residues: A, MBP−Cit_0; B, MBP−$Cit_{2.7}$; C, MBP−$Cit_{3.8}$; D, MBP−$Cit_{7.0}$.

for peptide 43−88 are shown. It can be seen that peptide 43−169 was released rapidly from the intact protein and the rate increased when MBP−$Cit_{7.0}$ was used. With both MBP−Cit_0 and MBP−$Cit_{2.7}$, the release of peptide 43−88 was slow. However, with MBP−$Cit_{7.0}$, the release of peptide was rapid, suggesting that 7.0 mol of citrulline in MBP altered the structure, increasing the accessibility of Phe−Phe linkage 88−89 to the enzyme. Since peptide 43−88 has been

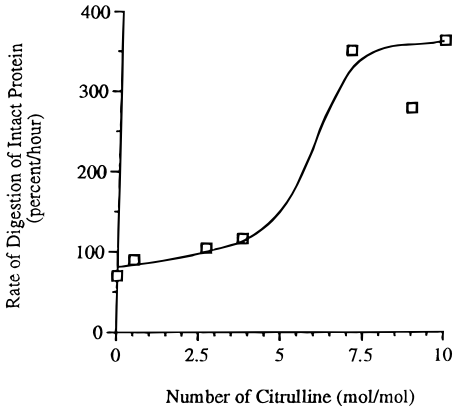

FIGURE 4: Rate of digestion of bMBP containing different numbers of citrullinyl residues. D: The intact protein was not detectable after 0.5 h.

reported to contain the immunodominant epitope (*12*, *13*), the rapid release of this peptide after deimination of 7.0 arginyl residues/mol of MBP suggests important roles for PAD and cathepsin D in generating this encephalitogenic peptide in demyelinating disease.

Molecular Dynamics Simulations of Human MBP−Cit$_x$ Models. The bovine MBP was used in the enzymatic studies to conserve human material. However, the molecular dynamics simulations were done with human MBP because this was the model available. The results of the molecular dynamics simulations of models of hMBP−Cit$_0$, hMBP−Cit$_6$, and hMBP−Cit$_{19}$ are shown in Figure 6. The protein hMBP−Cit$_0$ (human MBP) forms an extended C-shape primarily, with an antiparallel β-sheet forming the central core and loop regions on either side (Figure 6A−C). With hMBP−Cit$_6$ (Figure 6D), the C-shape is still apparent, but a definite structural rearrangement has taken place. The model of hMBP−Cit$_{19}$ (Figure 6E,F) is more open than either hMBP−Cit$_0$ or hMBP−Cit$_6$. The loop regions become more extended, and there are more subtle distortions in the β-sheet backbone that diminish its degree of curvature. On a local scale, the environments of the cathepsin D cutting sites at the two Phe−Phe linkages, in the middle of the β-sheet backbone, are not dramatically changed. However, the loop region containing Arg 97 (human sequence; bovine Arg 95) does undergo a dramatic shift that can be seen visually, and this movement must have some effect at the cathepsin D cutting sites.

This model is consistent with circular dichroism data reported earlier (*15*). At that time hMBP−Cit$_0$ was deiminated with PAD, resulting in the deimination of 17 of the 19 arginyl residues. A large increase in random structure (as measured by $[\theta]_{200}$) was observed. At 5 °C the $[\theta]_{200}$ of the deiminated protein was -70×10^3 compared to -30×10^3 deg·cm^2/dmol for hMBP−Cit$_0$. In a temperature study from 5 to 70 °C the ratio of $[\theta]_{200}$ for deiminated/undeiminated remained the same, i.e., approximately 2:1. These data substantiate that deimination of hMBP−Cit$_0$ generates a more open structure. Since CD is an averaging technique, the molecular dynamic simulations give a better picture of the effect of deimination on particular residues, e.g., Arg 97.

Quantitative comparisons of the three models were as follows. The root-mean-squared deviations of the protein backbones were 0.571 nm (hMBP−Cit$_0$ to hMBP−Cit$_6$), 0.821 nm (hMBP−Cit$_0$ to hMBP−Cit$_{19}$), and 0.769 nm (hMBP−Cit$_6$ to hMBP−Cit$_{19}$). The total surface accessibilities of all 170 residues were roughly 3020, 2970, and 3210 Å^2 for hMBP−Cit$_0$, hMBP−Cit$_6$, and hMBP−Cit$_{19}$, respectively. The same trend was followed for the total surface accessibilities of all 19 Arg/Cit residues or of the specified group of 6 Arg/Cit residues (residues 25, 31, 122, 130, 159, and 170) in the human sequence. These residues were most exposed in the most citrullinated form of the protein.

DISCUSSION

The presence of increased amounts of the citrullinated MBP−Cit$_6$ has been documented in established MS (*5*), in which it accounted for about 45% of the total MBP. An increased extent of citrullination was reported in fulminating MS, so that instead of 6 citrullinyl residues found in chronic MS, MBP component C-8 was shown to have 18 citrullines (*6*), and this MBP−Cit$_{18}$ accounted for more than 80% of the total MBP. The importance of the observation of 18 citrullines relies heavily on assurances that it is not artifactual, possibly due to post mortem autolysis. We have addressed this issue in the following way. In victims of chronic MS, MBP−Cit$_6$ accounted for about 45% of the total MBP, whereas it accounted for 20% in normal age matched controls who died of nonneurological causes. No change in the amount of MBP−Cit$_6$ was observed in Alzheimer's, Parkinson's, Huntington's, amyotropic lateral sclerosis, or motor neuron disease (*5*) compared to normal. The citrullinated MBP was observed in a biopsy sample obtained for intractable epilepsy by immunogold labeling of cryosections, suggesting that the citrullinated MBP was present in vivo (*16*). In the experimental allergic encephalomyelitis (EAE) model of MS, no change in the citrullinated MBP was observed in any cases of acute EAE of 2 months duration or in chronic EAE of 4 months or even 12 months duration. Therefore, neither acute nor chronic inflammatory pathology produced an increase in the amount or degree of citrullinated MBP (*17*). In rheumatoid arthritis (RA), a disease with many similarities to MS, autoantibodies in sera of RA patients only recognized epitopes in which the arginyl residues were deiminated to citrulline (*18*, *19*). Since all RA patients were alive, the conversion of arginine to citrulline could not be considered a post mortem artifact. Taken together, these data suggest that the conversion of arginine to citrulline represents an important event in the chemical pathogenesis of demyelinating disease in particular and of autoimmune disease in general.

Cathepsin D, a member of the metalloproteinase family, was shown to be elevated in brains from victims of MS (*10*). It was especially marked around active plaques (*11*). Peptide 45−89 (human sequence), produced by the digestion of MBP at the two Phe−Phe linkages, has been found in the cerebrospinal fluid of patients with MS (*12*). It is clear that cathepsin D has an important role in the chemical pathology in MS. Under normal circumstances, the enzyme does not have access to MBP in the compact myelin sheath. However, decreased binding of MBP to the acidic lipids of the myelin sheath by the deimination of arginyl residues of MBP by PAD allows the enzyme access to the substrate by disrupting the electrostatic protein−lipid interactions essential for myelin integrity. In addition, significant changes in three-dimensional structure resulting from the deimination further increase the susceptibility of MBP to cathepsin D.

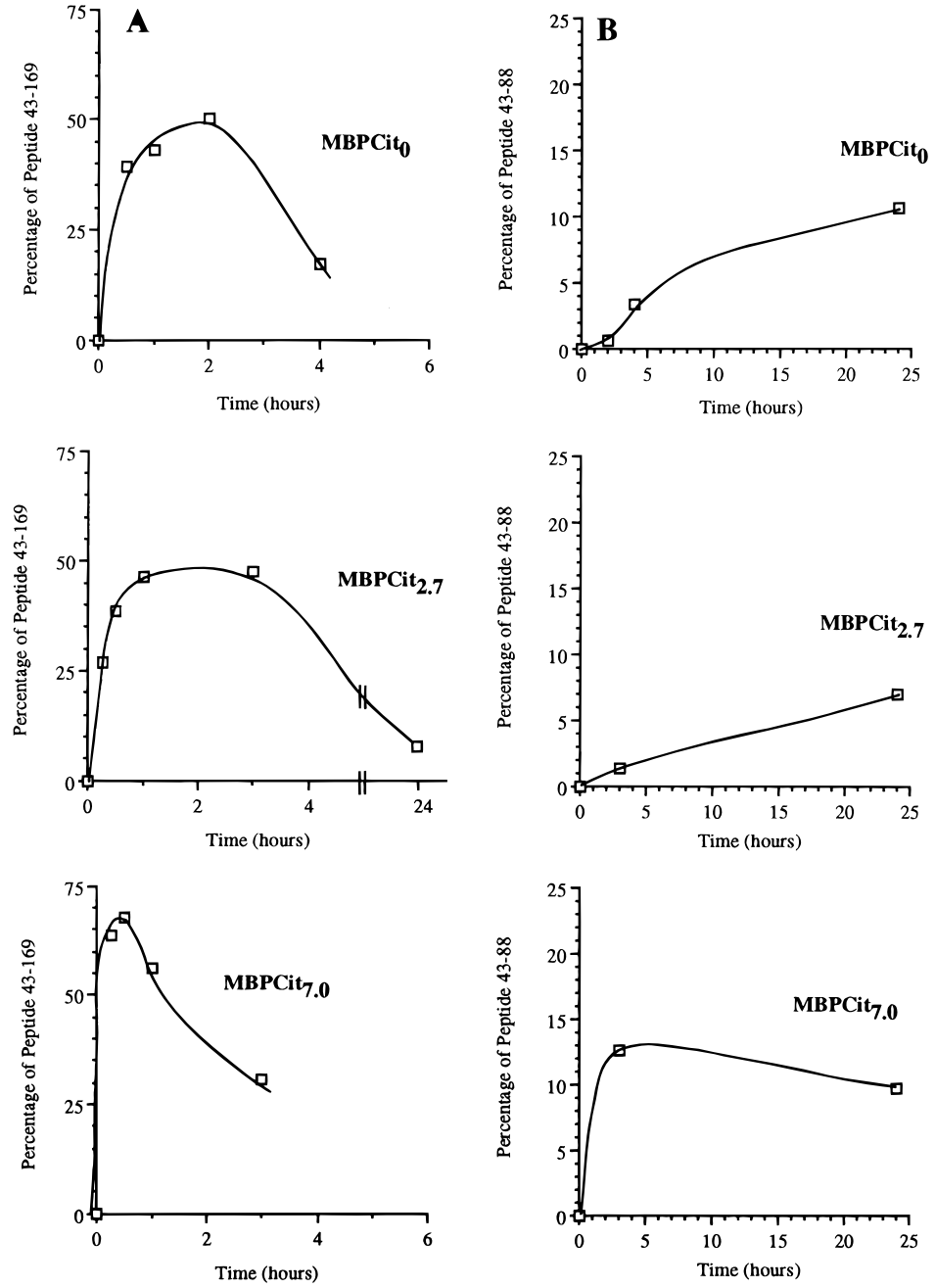

FIGURE 5: Percentage (peptide/total peptides × 100) of peptides 43−169 (panel A) and 43−88 (panel B) at different times in bMBP−Cit_0, bMBP−$Cit_{2.7}$, and bMBP−$Cit_{7.0}$.

The only three-dimensional atomic structure model of human MBP that is presently available is an ab initio prediction based on published reports of the dimensions of the proteins, phylogenetic sequence comparisons indicating conservation of β-strand-forming regions, and reconstructions from cryotransmission electron micrographs (*20*, *21*) (Protein Data Bank accession number 1qcl). At its present state of development, this model comprises a central antiparallel β-sheet backbone with extensive loop regions on either side and is bent into a "C"-shape to correspond to the three-dimensional reconstructions (Figure 6). The loop regions can easily accommodate α-helical segments when these become better defined (*22*). The whole protein can be straightened out to a length of roughly 15 nm, width of 4.5 nm, and height of 1.5 nm, to fit within the confines of the major dense line in myelin (*23*). Here, this protein model served as a tool to investigate the effects of citrullination of arginines as observed in MS. The model hMBP−Cit_6 is equivalent to charge component hMBP−C-8 in chronic MS, whereas hMBP−Cit_{19} is equivalent to charge component hMBP−C-8 in acute Marburg MS. The most striking structural change occurred with hMBP−Cit_{19}. The molecule became significantly more extended than in its less citrullinated forms, and the total surface accessibility of all residues also increased. This more open structure is entirely consistent with the circular dichroism data reported by us earlier (*15*). At that time we reported that the deimination of 17 of 19 arginyl residues of hMBP−Cit_0 resulted in a 2-fold change in unordered structure. Since CD is an averaging technique, the model in Figure 6 gives us some insight on how deimination affects the three-dimensional orientation of specific residues. Therefore, MBP and many other proteins

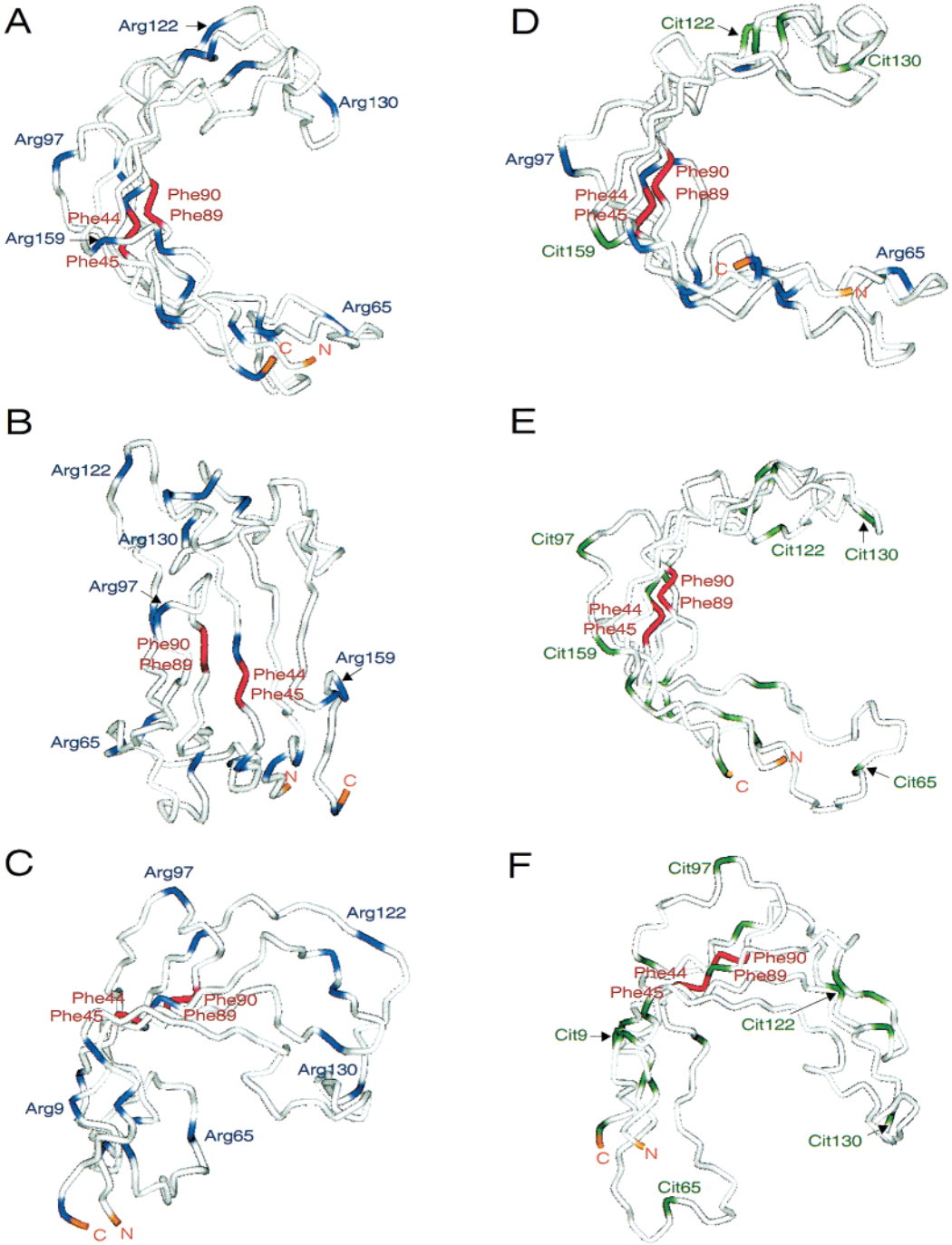

FIGURE 6: Three-dimensional structural models of hMBP−Cit_0 (A−C), hMBP−Cit_6 (D), and hMBP−Cit_{19} (E, F) following molecular dynamics and energy minimization. Selected Arg/Cit residues, cathepsin D cutting sites, and the C- and N-termini are shown. The structures are shown in comparable orientations in (i) panels A, D, and E and (ii) panels C and F. Panel B shows the five-stranded antiparallel β-sheet backbone.

(see introduction) undergo large changes in secondary structure as a result of deimination. Although the cathepsin D binding sites themselves did not become dramatically more exposed, the overall opening up of the molecule must have some effect on their accessibility or susceptibility to cathepsin D. Given the limitations of the atomic model and of the conditions of the molecular dynamics computations, these results are in agreement with cryotransmission electron microscopic reconstructions of these charge isomers (*8*), as well as with the digestion data presented here, and fully support the thesis that citrullination causes a dramatic conformational change in MBP.

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