Molecular dynamics simulation of thionated hen egg white lysozyme

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Abstract: Understanding of the driving forces of protein folding is a complex challenge because different types of interactions play a varying role. To investigate the role of hydrogen bonding involving the backbone, the effect of thio substitutions in a protein, hen egg white lysozyme (HEWL), was investigated through molecular dynamics simulations of native as well as partly (only residues in loops) and fully thionated HEWL using the GROMOS 54A7 force field. The results of the three simulations show that the structural properties of fully thionated HEWL clearly differ from those of the native protein, while for partly thionated HEWL they only changed slightly compared with native HEWL. The analysis of the torsional-angle distributions and hydrogen bonds in the backbone suggests that the α -helical segments of native HEWL tend to show a propensity to convert to 3_{10} -helical geometry in fully thionated HEWL. A comparison of the simulated quantities with experimental NMR data such as nuclear overhauser effect (NOE) atom–atom distance bounds and $^3J_{HNH_\alpha}$ -couplings measured for native HEWL illustrates that the information content of these quantities with respect to the structural changes induced by thionation of the protein backbone is rather limited.

Keywords: thio substitution; hen egg white lysozyme (HEWL); GROMOS; molecular dynamics simulation; hydrogen bonding

Introduction

Understanding the mechanism and driving forces of protein folding is one of the major challenges in molecular biology. The folding process is complicated because different types of forces and effects are involved, for example, Coulomb and van der Waals forces, the hydrophobic effect, hydrogen bonding, and so forth. There exist different viewpoints about the factors determining protein folding,^{1–5} in particular concerning the contribution of hydrogen bonding. To investigate the effect of hydrogen bonding on the stability of a protein fold one can remove

hydrogen-bond donors from the polypeptide backbone, as was done in molecular dynamics (MD) simulations of ester-linked hen egg white lysozyme (HEWL) by Eichenberger *et al.*^{6,7} HEWL is one of the most studied and best characterized globular proteins, and high-quality experimental data at the atomic level are available from X-ray diffraction⁸ and NMR spectroscopy.^{9–11} It is a 129-residue protein with as main secondary structure elements four α -helices, two 3_{10} -helices, and three β -sheets (Table I).

The replacement of the amide bond by a thioamide bond is a conservative modification of the peptide backbone (Fig. 1), which has been found to improve the bioactivity and stability of peptidomimetics.^{12,13} In this modification, the backbone hydrogen-bond acceptor oxygen is replaced by sulfur thereby reducing the hydrogen-bond forming capability of the backbone. Many computational and experimental studies on different thiopeptides have been reported in the past decade.^{14–19} To further investigate the effect of varying the strength and geometry of hydrogen bonds involving the backbone in protein folding, it will be of interest to see whether

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Table I. Residue Numbers of Residues Involved in Secondary Structure Elements of HEWL

α -Helices	3_{10} -Helices	β -Sheets
Helix A: 5–14	Helix a: 80–83	43–45
Helix B: 25–34	Helix b: 120–123	51–53
Helix C: 89–100		58–59
Helix D: 109–114		

partly or fully thionated HEWL is still able to maintain the structural properties of native HEWL.

In this study, three 20 ns MD simulations of native HEWL, partly thionated HEWL in which 74 amide bonds of residues not involved in any of the helices or β -sheets aforementioned are replaced by thioamide bonds, and fully thionated HEWL in which the 128 amide bonds between all residues are replaced by thioamide bonds, are described. The simulation results of these three proteins are analyzed, and their structural properties are compared. In addition, the simulation results are compared with experimental data, nuclear overhauser effect (NOE) atom–atom distances,¹¹ and 3J -couplings⁹ derived from NMR measurements of native HEWL.

Results and Discussion

Structural properties and flexibility

The root-mean-square deviations (RMSD) of the backbone atoms from the energy-minimized X-ray structure for the simulation trajectories of native, partly and fully thionated HEWL are shown in Figure 2, upper panel. For all three proteins, the RMSD values increase during the first 7 ns and level off around 0.27 nm for native HEWL and about 0.35 nm for partly and fully thionated HEWL, indicating that the positions of the backbone atoms of the thionated proteins deviate slightly more from the energy-minimized X-ray structure of native HEWL than the backbone atoms of the native HEWL simulation. The observation that the properties shown in Figure 2 do not significantly change during the last 13 ns of simulation suggests that the simulations are sufficiently long to obtain an impression of the structural changes induced by thionation.

The radius of gyration, a measure of compactness of the structure, calculated for native, partly and fully thionated HEWL is shown in Figure 2, middle panel. Native and partly thionated HEWL are more compact than fully thionated HEWL. This

Figure 1. Thio substitution in a peptide bond.

Figure 2. Upper panel: Time evolution of atom-positional RMSD from the initial X-ray structure²⁰ of the backbone atoms (N, C α , C, O/S). Middle panel: Time evolution of the radius of gyration of backbone atoms. Lower panel: Time evolution of the SASA. Black: native HEWL; red: partly thionated HEWL; purple: fully thionated HEWL.

trend is also observed in the solvent accessible surface area (SASA) (Fig. 2, lower panel). It may be explained by the larger size of the sulfur atom compared with the oxygen atom and the longer hydrogen-bond length of a hydrogen bond involving the C=S moiety.

The time evolution of secondary structure elements and the atom-positional root-mean-square fluctuations (RMSF) of the C α atoms in the three simulations are shown in Figure 3. Native and partly thionated HEWL largely maintain the major secondary structure elements during the 20 ns simulation except for the α -helix D (residues 109–114), which converts to a π -helix. Fully thionated HEWL shows slightly larger atom-positional RMSF than partly thionated and native HEWL (right panels), yet the major secondary structure elements did not totally disappear in the simulation, see Figure 4.

The ϕ/ψ angle distributions of the residues involved in the four α -helices and two 3_{10} -helices calculated from the simulation trajectories of native HEWL, partly thionated and fully thionated HEWL are shown in Figure 5. The angle distributions in native HEWL and partly thionated HEWL are similar, while in fully thionated HEWL the distribution of the ϕ angle is shifted to more negative values and the distribution of the ψ angle is shifted to less negative values. This change is due to a structural accommodation to the longer N–H...S=C hydrogen bonds, see also Ref. 18.

The occurrences of backbone–backbone, backbone–side chain, and side chain–side chain hydrogen bonds present in the native, partly thionated, and

Figure 3. Secondary structure elements as a function of time (left panels) and the atom-positional RMSF of the C α atoms (right panels) for three MD simulation trajectories of native (upper panels), partly thionated (middle panels), and fully thionated HEWL (lower panels).

fully thionated HEWL simulations are listed in Supporting Information Tables SI–SIII. Hydrogen bonds with an occurrence lower than 10% are not listed in these tables. The number of hydrogen bonds, inter- or intra-helical or between residues of β -sheets, as well as the cumulative sum over the corresponding occurrences for the three simulations is summarized in Table II. We see that for fully thionated HEWL, the number of inter- or intra-helical backbone–backbone hydrogen bonds increases significantly, but the corresponding cumulative sum of the occurrences increases only little. The number of inter- or intra-helical backbone–side chain hydrogen bonds of fully thionated HEWL also increases, as well as their occurrences. For the hydrogen bonds between residues in β -sheets, the numbers of backbone–backbone

and side chain–side chain hydrogen bonds are the same for all three simulations, while the occurrences in fully thionated HEWL differ from those in the other two simulations. There are no backbone–side chain hydrogen bonds with a higher occurrence than 10% found in the simulations of native or partly thionated HEWL, but two were found in the simulations of fully thionated HEWL.

An analysis of *i* to *i*-4, *i* to *i*-3 and *i* to *i*-2 backbone–backbone hydrogen bonds in the loops where *i* denotes the residue sequence number shows that full thionation increases *i* to *i*-3 hydrogen bonding at the expense of *i* to *i*-4 hydrogen bonding, in particular for residues 37, 107, and 108.

Next we focus on the intra-helical (Table I) backbone–backbone hydrogen bonds. The number

Figure 4. Schematic diagrams showing a superposition of different structures of HEWL: (a) the X-ray structure of native HEWL (grey) and of native HEWL after 20 ns of simulation (cyan), (b) the structure of native HEWL after 20 ns of simulation (cyan) and of partly thionated HEWL after 20 ns of simulation (orange), (c) the structure of native HEWL after 20 ns of simulation (cyan) and of fully thionated HEWL after 20 ns of simulation (purple), and (d) the structure of partly thionated HEWL after 20 ns of simulation (orange) and of fully thionated HEWL after 20 ns of simulation (purple).

and occurrence of hydrogen bonds of type NH(i)-O(i-4) or NH(i)...S(i-4) in the four segments that show α -helical structure in the X-ray structure and hydrogen bonds of type NH(i)...O(i-3) or NH(i)...S(i-3) in the four segments that show α -helical and two

segments that show 3_{10} -helical structure in the X-ray structure are listed in Table III. The number and occurrence of hydrogen bonds of type NH(i)-O(i-4) or NH(i)...S(i-4) in the simulations of native and partly thionated HEWL are similar, but

Figure 5. The ϕ/ψ angle distributions of six helices based on the 20 ns simulation trajectories of native HEWL (black), partly thionated HEWL (red), and fully thionated HEWL (purple). The sequence numbers of the residues involved in helical structures in the X-ray structure of native HEWL are specified in the panels.

Table II. Number and Occurrence of Hydrogen Bonds, Inter- or Intra-Helical or Between β -Sheet Residues

	Backbone–backbone		Backbone–side chain		Side chain–side chain	
	Number	Occ. (%)	Number	Occ. (%)	Number	Occ. (%)
Helix–helix						
Native HEWL	27	1936	6	161	2	27
Partly thionated HEWL	28	1930	8	236	0	0
Fully thionated HEWL	51	2183	17	399	1	43
Sheet–sheet						
Native HEWL	4	353	0	0	4	136
Partly thionated HEWL	4	356	0	0	4	195
Fully thionated HEWL	4	289	2	44	4	158

Hydrogen bonds with an occurrence lower than 10% are not considered. The occurrence is the cumulative sum over the corresponding number of hydrogen bonds, the full list is given in Supporting Information Tables SI–SIII.

decrease in the simulation of fully thionated HEWL. The number and occurrence of hydrogen bonds of type NH(i)...O(i-3) or NH(i)...S(i-3) increases a bit in the simulation of partly thionated HEWL compared with those in the simulation of native HEWL, while they increase much more in the simulation of fully thionated HEWL. It shows that more hydrogen bonds of i to i-3 type, that is, short-ranged along the sequence, are formed in the simulation of fully thionated HEWL.

To further study hydrogen bonding in the helical segments A to D (Table I), we analyzed the distributions of the distance between hydrogen (H) and acceptor atom (O or S) and angles N...H...O or N...H...S in the four helical segments (Figs. 6 and 7). In Figure 6, we see that the distributions of NH(i)...O/S(i-4) and NH(i)...O/S(i-3) distances in the simulation of native and partly thionated HEWL are rather similar, while the distributions of NH(i)...O/S(i-4) distances in the simulation of fully thionated HEWL shift to larger values. The peaks of the distributions of NH(i)...O/S(i-4) distances in the simulation of fully thionated HEWL are close to the peaks of the distributions of NH(i)...O/S(i-3) distances. In Figure 7, it is shown that the distributions of NH(i)...O/S(i-4) angles significantly shift to lower

values while the distributions of NH(i)...O/S(i-3) angles shift to larger values except in the helical segment D in the simulation of fully thionated HEWL compared with the other two simulations. It explains why there are less NH(i)...O/S(i-4) hydrogen bonds and more NH(i)...O/S(i-3) hydrogen bonds formed in the four helical segments in the simulation of fully thionated HEWL than in the simulations of native and partly thionated HEWL. Apparently, thionation of α -helical segments of the polypeptide backbone favors the occurrence of i to i-3 compared with i to i-4 backbone–backbone hydrogen bonds.

Comparison with experimental NMR data

The proton–proton NOE distance bound violations with respect to the experimentally derived values¹¹ were calculated for native HEWL, partly thionated and fully thionated HEWL. Two sets of NOE bounds for native HEWL were used, one set includes 1630 NOE bounds that are available from NMR experiments,¹¹ the other set contains 392 long-range bounds between atoms in residues *i* and *j* with $j \geq i + 4$ only. The number of violations of all NOE distance bounds and of long-range bounds only are given in Table IV, while the distributions of all NOE distances

Table III. Number and Occurrence of Intra-Helical Backbone–Backbone Hydrogen Bonds

	Native HEWL		Partly thionated HEWL		Fully thionated HEWL	
	Number	Occ. (%)	Number	Occ. (%)	Number	Occ. (%)
NH(i)...O(i-4)/NH(i)...S(i-4)						
Helix A	6	565	6	574	6	266
Helix B	6	480	6	523	6	468
Helix C	8	631	8	561	6	264
Helix D	2	80	2	57	2	73
NH(i)...O(i-3)/NH(i)...S(i-3)						
Helix A	0	0	0	0	6	235
Helix B	2	34	1	21	4	112
Helix C	1	24	2	58	9	403
Helix D	0	0	1	55	2	70
Helix a	1	51	1	65	1	68
Helix b	0	0	1	16	1	34

Hydrogen bonds with an occurrence lower than 10% are not considered. The occurrence is the cumulative sum over the corresponding numbers of hydrogen bonds.

Figure 6. Distance distributions of NH(i)...O/S(i-4) (upper panels) and NH(i)...O/S(i-3) (lower panels) of four helical segments A–D in the residue sequence in simulations of native HEWL (black line), partly thionated HEWL (red line) and fully thionated HEWL (purple line).

are shown as bound violations in Figure 8 (left panels). The total number of violations in partly thionated HEWL increases substantially compared with that in native HEWL. This number increases even more in fully thionated HEWL. For the long-range violations, the number also increases from native HEWL to partly thionated HEWL to fully thionated HEWL. Most of the medium (> 0.3 nm and < 0.5 nm) violations and all the large (> 0.5 nm) ones involve long-range bounds. Interestingly, the number of large long-range violations in partly thionated HEWL is larger than in fully thionated HEWL. The detailed NOE distance bounds and bound violations (> 0.1 nm) for native, partly thionated and fully thionated HEWL are listed in Supporting Information Table SIV. The structural changes in HEWL upon partial or full thionation of the peptide moieties are only

weakly reflected in an increase of the number of NOE distance bound violations.

The experimental and calculated $^3J_{\text{H}_\text{N}\text{H}_\alpha}$ -coupling constants are shown in Figure 8 (right panels). The RMSD of the calculated coupling constants from the experimental values are 1.7, 1.6, and 1.8 Hz for native, partly thionated and fully thionated HEWL, respectively. Thus, the structural changes upon thionation are only weakly reflected in the overall agreement. The detailed $^3J_{\text{H}_\text{N}\text{H}_\alpha}$ -coupling constants for the HEWL from NMR experiments and averaged over MD trajectories are listed in Supporting Information Table SV.

Conclusions

To understand the effect of thio substitutions in the peptide bonds upon the structure of a protein, we

Figure 7. Angle distributions of NH(i)...O/S(i-4) (upper panels) and NH(i)...O/S(i-3) (lower panels) of four helical segments in the residue sequence in simulations of native HEWL (black line), partly thionated HEWL (red line) and fully thionated HEWL (purple line).

Table IV. NOE Distance Bound Violations in Simulations of Native, Partly Thionated and Fully Thionated HEWL With Respect to the Experimental NMR NOE Distance Bounds of Native HEWL¹¹

	Number of NOE bound violations (all/long-range)		
	>0.1 nm	>0.3 nm	>0.5 nm
Native	59/38	11/8	0/0
Partly thionated	66/50	15/15	6/6
Fully thionated	96/71	18/17	2/2

Two sets of NOE bounds have been used: one includes 1630 NOE bounds, the other includes the 392 long-range (residues i and j with $j \geq i + 4$) NOE bounds only.

separately simulated native HEWL, partly (57%, only residues not involved in secondary structure) thionated HEWL and fully thionated HEWL in aqueous solution for 20 ns using the GROMOS 54A7 force field. The result shows that fully thionated HEWL is slightly less compact than the native and partly thionated ones. The major secondary structure elements are not completely lost in the simulation of fully thionated HEWL. The helical segments of native HEWL have more negative ϕ angles and less negative ψ angles in fully thionated HEWL compared with native or partly thionated HEWL. The analysis of hydrogen bonding shows that thionation of the backbone tends to convert α -helical structures into more 3_{10} -helical structures with very similar i to $i-4$ and i to $i-3$ hydrogen-bond lengths and angles.

A comparison of the simulation results with experimental NMR data of native HEWL shows that the number of violations of NOE distance bounds measured for native HEWL increases from native

HEWL to partly thionated HEWL to fully thionated HEWL, but is still remarkably low due to the compactness of the simulated structures. The overall agreement of the calculated $^3J_{\text{H}_\text{N}\text{H}_\alpha}$ -coupling constants with the ones measured for native HEWL does not change much in the three simulations, which illustrates the low information content with respect to structure of these data which may be due to the approximative nature of the Karplus relation.

Computational Methods

Peptide substitutions

In partly thionated HEWL, the backbone carboxyl group of residues 1–4, 15–24, 35–42, 46–50, 54–57, 60–79, 84–88, 101–108, 115–119, and 124–128 was replaced by a C=S group. In fully thionated HEWL, all residues except residue 129 were thionated.

Parameters

The force-field parameters for the C=S group were chosen as to be compatible with the GROMOS 54A7 parameter set.^{21,22} Since the electronegativity of sulfur is less than that of oxygen, the charge of S was defined to be $-0.20 e$ (compared with $-0.45 e$ for a carboxyl oxygen), and the charge of the corresponding carboxyl C atom was changed to $+0.20 e$. It is reported that the C=S bond is 37% longer than the C=O bond,¹⁹ that is, the C=S bond length was taken as 0.163 nm (bond type 29 in Table 2 of Ref. 21). The 54A7 van der Waals parameters for C and S were kept unchanged, that is, the GROMOS integer atom codes of atoms C and S remained 12 and 23, respectively (Tables 6 and 7 of Ref. 21), and the

Figure 8. Occurrence of $\langle r^{-3} \rangle^{-1/3}$ averaged NOE distance bound violations (left panels) and comparison of averaged $^3J_{\text{H}_\text{N}\text{H}_\alpha}$ -values as obtained from simulations and experimental data. Upper panels: native HEWL; middle panels: partly thionated HEWL; lower panels: fully thionated HEWL.

pairwise interaction selection (Table 8 of Ref. 21) remained unchanged. The mass codes (Table 1 of Ref. 21) of C and S remained 12 and 32. Bond-angle, improper dihedral-angle, and torsional dihedral-angle parameters were all kept as for a peptidic C=O. Thus, we only investigate the effects of a change in partial charge, van der Waals parameters, and bond length of this moiety.

Simulations

The MD simulations of native, partly thionated, and fully thionated HEWL were all performed using the GROMOS^{23–25} simulation package and the GROMOS force-field parameter set 54A7.²²

The initial structures were derived from a native crystal structure²⁰ by replacing the amide oxygen atom by a sulfur atom in the loop residues to obtain partly thionated HEWL or in all but the last residues to obtain fully thionated HEWL, and each protein was solvated in a periodic, cubic box with 14,365, 19,550, or 19,551 SPC water molecules,²⁶ respectively. The minimum protein-box wall distance was set to 1.8 nm for all three simulations. The protonation states of protonizable groups were selected to correspond to a pH of 7. Eight chloride ions were added to achieve overall neutrality of the system.

All three simulations were performed for 20 ns at a constant temperature of 300 K and a constant pressure of 1 atm using the weak coupling algorithm.²⁷ The temperature coupling time was set to 0.1 ps, the pressure coupling time to 0.5 ps, and an isothermal compressibility of $4.575 \times 10^{-4}(\text{kJ mol}^{-1}\text{nm}^{-3})^{-1}$ was used.²⁸ The protein and the solvent were separately coupled to a heat bath.

All bond lengths and the bond angles of the water molecules were kept rigid by applying constraints using the SHAKE algorithm,²⁹ allowing for a time step of 2 fs in the leap-frog algorithm to integrate the equations of motion. Nonbonded interactions were calculated using a triple-range cutoff scheme with cutoff radii of 0.8/1.4 nm. Interactions within 0.8 nm were evaluated every time step, the intermediate range interactions were updated every fifth time step and the long-range electrostatic interactions beyond 1.4 nm were approximated by a reaction field force³⁰ representing a dielectric continuum with a dielectric permittivity of 61.³¹ Configurations of the system were saved every 5 ps for analysis.

Analysis

The atom-positional RMSD between pairs of structures were evaluated based on all backbone atoms (native: N, CA, C, O; thionated: N, CA, C, S) of all 129 residues. The atom-positional RMSF were also calculated for all 129 residues but only considering the CA atoms. A translational superposition of the solute centers of mass and least-squares rotational fitting with respect to the crystal structure were

applied in both RMSD and RMSF calculations. The SASA was calculated using the algorithm proposed by Lee and Richards.³² The radius of gyration of the protein, a measure of the compactness of the structure, was calculated using the definition,

$$R_{\text{gyr}} = \left[\frac{1}{N_a} \sum_{i=1}^{N_a} (\vec{r}_i - \vec{R}_{\text{cm}})^2 \right]^{1/2} \quad (1)$$

with

$$\vec{R}_{\text{cm}} = M^{-1} \sum_{i=1}^{N_a} m_i \vec{r}_i \quad (2)$$

and

$$M = \sum_{i=1}^{N_a} m_i \quad (3)$$

where $\vec{r}_i$ denotes the Cartesian position vector of atom i , m_i is its mass, and N_a denotes the number of protein atoms considered.

Hydrogen bonds were analyzed according to a geometric criterion: a minimum donor-hydrogen-acceptor angle of 135° and a maximum hydrogen-acceptor distance of 0.25 nm for the N–H...O=C hydrogen bonds²⁸ and 0.35 nm for the N–H...S=C hydrogen bonds in view of larger size of the C atom.³³ Secondary structure assignments were performed using the rules defined by Kabsch and Sander.³⁴

1630 proton-proton distances extracted from the NOE intensities measured in the NMR experiments for native HEWL¹¹ were compared with the average proton-proton distances in the simulations calculated using $\langle r^{-3} \rangle^{-1/3}$ averaging, where r is the instantaneous proton-proton distance. The experimentally derived 3J -coupling constants measured by Smith *et al.*⁹ were used for comparison.

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