Structural Consequences of Reductive Methylation of Lysine Residues in Hen Egg White Lysozyme: An X-ray Analysis at 1.8-Å Resolution†

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ABSTRACT: Chemical modification of proteins has been and continues to be an important biochemical tool for the study of protein structure and function. One such type of approach has been the reductive methylation of lysine residues. In order to address the consequences of such methylation on the crystallization and structural properties of a protein, the three-dimensional structure of hen egg white lysozyme in which all lysine residues have been alkylated has been determined and refined to a nominal resolution of 1.8 Å and a crystallographic R factor of 17.3%. Crystals used in the investigation were grown from 1.5–1.8 M $MgSO_4$ and 50 mM Tris at pH 8.0 and belonged to the space group $P2_12_12_1$ with unit cell dimensions of a = 30.6 Å, b = 56.3 Å, c = 73.2 Å, and one molecule per asymmetric unit. It was not possible to grow crystals of the modified lysozyme under the conditions normally employed for the hen egg white protein. Overall, the three-dimensional structures of the native lysozyme and the modified protein are very similar with only two surface loops differing to any significant extent. Specifically, the positions of the α-carbons for these two forms of the protein, excluding the surface loops, superimpose with a root-mean-square value of 0.40 Å. The magnitude of the structural changes observed between the modified and unmodified forms of lysozyme is similar to that seen when an identical protein structure is solved in two different crystalline lattices. Consequently, the methylation of lysine residues results in very little structural perturbations but can produce enormous effects on the crystallization properties of a protein. As described here, this technique was absolutely critical for obtaining X-ray quality crystals of myosin subfragment 1 and thus may prove to be valuable in the crystallization of other proteins that have so far resisted forming ordered arrays.

Chemical modification has proven to be one of the most powerful tools available for studying the biochemical properties of proteins. Historically, this approach has made many pivotal contributions to our understanding of protein structure and function (Means & Feeney, 1971). In most of these chemical labeling investigations, an effort has been made to change a specific amino acid residue with the goal of determining both its role and location in the protein of interest. In some instances, however, the results from such studies have been compromised due to nonspecific secondary reactions of the modification procedures. With the advent of site-directed mutagenesis, however, it has now become possible to exercise exquisite control over the nature and location of a change to an amino acid. Although this more recent approach offers obvious advantages, it still requires considerable prior knowledge of the protein's structure and function in order to perform meaningful experiments that can be readily interpreted. In contrast, the chemical labeling approach has provided significant information without such prior knowledge and has thus helped to define both the function and location of important amino acid residues. An example of this has been the use of diisopropylfluorophosphate to indicate the presence of reactive serine residues in the serine proteases.

In contrast to the chemically labeling of a specific residue, complete modification of a class of amino acids has proven generally less useful as a determinant of enzyme function since the results have been more difficult to analyze. As reported in this paper, however, reductive methylation of lysine residues offers the opportunity to change the surface properties of a protein and potentially its crystallization properties as well. Indeed, by this approach, it has been possible to grow crystals of some proteins that have resisted crystallization for many years including myosin subfragment 1, whose three-dimensional structure has been recently determined (Rayment et al., 1993).

The original crystals of myosin subfragment 1, although enormously exciting, were not suitable for a detailed crystallographic analysis due to their limited diffraction (4.5 Å) and to their high mosaic spread (Rayment & Winkelmann, 1984). It was concluded that the problems with these crystals were due both to the inherent heterogeneity of the proteolytic subfragment and to the nonspecific nature of the initial chemical modification scheme. In order to develop an improved methylation protocol for the myosin subfragment 1, a parallel study of chemical modification of hen egg white lysozyme was undertaken. From these studies, a large scale modification protocol was subsequently developed that yielded a homogeneous and reproducible product. In addition, the chemical labeling of lysozyme allowed the effects of reductive alkylation on both the crystallization conditions and the molecular structure of the protein to be examined. As such this investigation of lysozyme provided a benchmark for the crystallographic studies of myosin subfragment 1 by addressing the structural consequences of reductively methylating lysine residues.

Hen egg white lysozyme was chosen for this investigation for several reasons. First of all, it has been one of the most thoroughly characterized enzymes and as such has played a

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central role in the development of our understanding of protein structure, enzyme function, protein folding, and protein stability. It has been the subject of an enormous number of chemical and physical studies and, indeed, was the first enzyme to be studied by X-ray crystallographic techniques (Blake et al., 1965). In addition, its structure has been determined and refined to high resolution in several crystalline forms, and these crystal structures have demonstrated that differing lattice contacts produce only small changes in the overall architecture of the molecule (Joynson et al., 1970; Moult et al., 1976; Artymiuk et al., 1982; Rao et al., 1983; Berthou et al., 1983; Kundrot & Richards, 1987; Kodandapani et al., 1990; Kachalova et al., 1991). Also, pure protein is commercially available in large quantities at a modest cost. Finally, lysozyme has been one of the easiest proteins to crystallize and consequently has been a frequent model for studying protein crystal growth and for developing crystallization protocols both on earth and in space (Littke & John, 1986; DeLucas et al., 1986).

At the time the crystals of myosin subfragment 1 were grown there was no precedent for the use of chemical modification as a crystallization technique. Thus it was essential to establish that no major changes in the secondary or tertiary structure were produced upon alkylation of lysine residues. Here we describe the chemical labeling protocols used for lysozyme, the crystallization and structure determination of the methylated protein to 1.8-Å resolution, and finally the comparison of the modified molecule with native lysozyme.

MATERIALS AND METHODS

Reductive Methylation of Lysine Residues. Sodium borohydride and dimethylamine borane complex were obtained from Aldrich Chemical Co. A 1 M stock solution of these compounds was prepared in water immediately prior to use. Formaldehyde was purchased as a 16% solution from Electron Microscopy Sciences and was diluted with water to form a 1 M stock solution immediately before use. A single batch of formaldehyde was used for all modifications of both lysozyme and myosin subfragment 1. All other chemicals were reagent grade and purchased through VWR.

The degree of labeling of the lysine residues was followed with a modification of the trinitrobenzene sulphonate (TNBS) colorimetric assay as described by Habeeb (1966). In this assay, 50 μL of the test solution was mixed with 50 μL of a solution containing 100 mM sodium borate, 40 mM potassium iodate, and 40 mM potassium iodide, pH 8.5, and incubated for 15 min at 37 °C. This step removed the excess reducing agent that interfered with the subsequent formation of the colored adduct. Following incubation, 900 μL of a 1% solution of TNBS at pH 8.5 was added and incubated at 37 °C for 30 min. The reaction was quenched with the addition of 1 mL of 10% SDS and 1 mL of 0.5 M HCl and the absorbance measured at 420 nM. The concentration of free primary amines present in the original sample was estimated from the molar extinction coefficient of 1.4×10^4 M^{-1} cm^{-1}.

The consumption of formaldehyde during the reductive alkylation was monitored by a colorimetric assay based on its reaction with 2,4-pentanedione in the presence of ammonium ions (Nash, 1953). In this assay, an aliquot of the reaction mixture containing up to 2 μmol of formaldehyde was added to 5 mL of a solution composed of 15 g of ammonium acetate, 0.3 mL of glacial acetic acid, 0.2 mL of 2,4-pentanedione, and 50 mL of methanol made up to a final volume of 100 mL. The assay mixture was incubated at 37 °C for 1 h and its absorbance recorded at 412 nm. The concentration of formaldehyde was estimated from a molar extinction coefficient of 18 000 M^{-1} cm^{-1}. The use of 50% methanol in the reaction mixture enhanced the solubility of the colored product and extended the range of the assay.

All other experiments were performed at 4 °C except where noted below. All solutions were prepared with reagent grade water.

Amino Acid Analyses. These were performed by Dr. Liane Mende-Mueller at the Amino-Acid and Nucleic-Acid Analysis Facility, Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226. Protein samples were exhaustively dialyzed prior to analysis against a 25 mM 4-ethylmorpholine–acetate buffer solution at pH 8.0.

Table I

amino acid	theoretical number	unmodified control, number[a]	sodium borohydride, number[a]	dimethyl amine–borane complex, number[a]
Cys	6	6.8	6.8	6.8
Asx	21	22.1	22.2	22.1
Thr	7	7.1	7.1	7.1
Ser	10	9.3	9.4	9.4
Glx	5	5.6	5.7	5.7
Pro	2	2.2	2.1	2.1
Gly	12	12.4	12.5	12.4
Ala	12	11.9	12.2	12.2
Val	6	6.2	5.7	5.9
Met[b]	2	1.8	1.9	1.9
Ile	6	6.1	6.1	6.1
Leu	8	8.8	8.9	8.8
Tyr[b]	3	2.0	2.2	1.9
Phe	3	3.0	3.1	3.1
His	1	1.0	1.0	1.0
Lys	6	6.1	0.0	0.0
MMLys		0.0	0.0	0.0
DMLys		0.0	5.5	5.6
Arg	11	11.6	11.8	11.7

[a] Based on 121 amino acid residues. Prior to hydrolysis the protein was oxidized with performic acid in order to estimate the cysteine content. As a conseqeunce no measurement was obtained for the methionine residues from this run. [b] The estimate of the methionine content given in this table was obtained from a separate hydrolysis of the same protein samples without the oxidation step. In the latter analysis, the estimate for the number of tyrosine residues was 3.1, 3.2, and 3.2 for the control, sodium borohydride, and dimethylamine borane complex reduced samples. These analyses did not include an estimate of the number of the tryptophan residues since these were lost during the hydrolysis of the protein. These analyses were performed by Dr. Liane Mende-Mueller at the Amino-Acid and Nucleic-Acid Analysis Facility, Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226. It is of interest that the number of dimethyllysine residues detected in the modified protein is lower than that observed in the native protein. This is due to methylation of the N-terminal amino group of the protein, which happens to be a lysine residue. The methylated N-terminal amino acid would not be detected in the present analysis; however, there is clear evidence in the electron density map for additional methyl groups on the N-terminal amino group.

RESULTS

Reductive Alkylation Protocol. Reductive alkylation of lysine residues involves the initial formation of a Schiff base between the ε-amino group of a lysine residue and either a ketone or aldehyde that is then reduced to form a secondary or tertiary amine as indicated for formaldehyde in eq 1.

$$R\text{–}NH_2 + CH_2O \rightleftharpoons R\text{–}N{=}CH_2 + H_2O \xrightarrow{\text{reduction}} R\text{–}NH\text{–}CH_3 \quad (1)$$

In the case of formaldehyde, the reaction proceeds rapidly to form the dimethyllysine product since the pK_a of the monomethyllysine residue is lower than that of lysine itself.

Table II: Intensity Statistics for the Methylated Lysozyme Crystal

	resolution range (Å)											
	∞ to 5.80	4.10	3.35	2.90	2.60	2.37	2.20	2.05	1.94	1.84	1.75	1.68
no. of measurements	1695	4266	5285	5918	6096	5775	4968	2697	2443	2080	1516	467
no. of independent reflections[a]	379 (309)	646 (584)	804 (762)	959 (932)	1075 (1059)	1183 (1119)	1265 (1029)	1322 (581)	1353 (517)	1318 (434)	1134 (293)	372 (83)
percentage of the theoretical no. of reflections	88.0	90.5	90.6	90.8	95.1	91.7	94.5	84.1	92.6	79.0	61.8	22.1
average intensity	3074	4130	3374	1834	1084	720	552	385	228	142	83	67
sigma	102	152	134	76	50	39	33	26	22	18	17	18
R_{factor} (%)[b]	1.9	2.2	2.3	2.6	3.0	3.7	4.2	4.7	6.8	9.3	14.4	18.4

[a] This is the number of reduced observations. Shown in parentheses is the number of measurements for which there were duplicate or symmetry-related measurements. [b] $R_{factor} = \sum|I - \bar{I}|/\sum I$.

The initial attempts to alkylate the lysine residues in myosin subfragment 1 used sodium cyanoborohydride as the reducing reagent (Rayment, unpublished results). Previous studies had demonstrated this reductant to be ideal for the labeling of proteins (Jentoft & Dearborn, 1979) since, in contrast to sodium borohydride (Means & Feeney, 1968), it was stable at pH 7.0. Consequently, many of the side reactions associated with the use of formaldehyde at higher pH could be avoided. Unfortunately, sodium cyanoborohydride ultimately proved unsuitable for the crystallization of myosin subfragment 1 due to the inherent reaction of the cyanide ion with the initial Schiff base formation as indicated in eq 2 (Gidley & Sanders, 1982).

$$R\text{–}N{=}CH_2 + CN^- + H^+ \rightarrow R\text{–}NH\text{–}CH_2CN \xrightarrow{\text{acid pH}} R\text{–}NH_3^+ + CH_2O + CN^- \quad (2)$$

This side reaction reduced the incorporation of formaldehyde into the final modified protein by more than 20% depending upon the concentration of sodium cyanoborohydride (Jentoft & Dearborn, 1979; Gidley & Sanders, 1982) and also presented three unacceptable problems in the preparation of material for crystallographic studies. First of all, it resulted in a heterogeneous population of molecules. Secondly, the slow hydrolysis of the *N*-cyanomethyl side product resulted in the release of formaldehyde which then reacted during the crystallization experiments with other amino acid side chains such as cysteines and histidines. Finally, formation of the Schiff base between lysine and formaldehyde also facilitated the modification of adjacent tyrosine, tryptophan, histidine, asparagine, and cysteine residues (French & Edsall, 1945). Consequently, in the early crystallization trials with modified myosin subfragment 1, there were large amounts of amorphous precipitate in the crystallization vials. The resulting crystals were small and displayed a high degree of mosaicity in their diffraction patterns. In light of these problems, two alternative reduction strategies were attempted.

Sodium Borohydride as the Reducing Agent. The next attempts to obtain complete modification of the lysine residues utilized sodium borohydride as the reducing agent following a protocol similar to that described by Means and Feeney (1968). Both myosin subfragment 1 and hen egg white lysozyme were modified in parallel experiments using this reagent. For the structural investigation described in this report, lysozyme at 10 mg/mL was initially dialyzed against 200 mM sodium borate buffer, pH 8.5. The reaction was conducted at pH 8.5 due to the instability of sodium borohydride at lower pH. The reduction was performed by adding in rapid succession 30 μL of 1 M formaldehyde followed by 6 μL of 1 M sodium borohydride per milliliter of protein with rapid stirring at 0 °C. After 10 min, an additional 3 μL of sodium borohydride was added. This procedure was repeated six times, with 30-min time intervals between the additions of formaldehyde, during which the reaction solution was left on ice. After the last addition, the mixture was allowed to sit for 30 min whereupon an additional 6 μL of 1 M sodium borohydride per milliliter of initial protein was added. The mixture was left on ice for one more hour after which the reaction was terminated by the addition of 0.5 g of solid ammonium sulfate per milliliter which served both to lower the pH and precipitate the protein. The precipitate was recovered by centrifugation at 19K in a Beckman JA20 rotor for 20 min (44000*g*) after which it was resuspended with water and dialyzed against 20 mM HEPES, pH 7.0, for 24 h. The final protein concentration was estimated from its absorbance at 280 nm with an A_{280} 0.1% of 2.64. As indicated in Table I, the amino acid analysis of the modified lysozyme showed essentially complete modification. This material was then crystallized and its structure determined as described in this report.

Table III: Refinement Statistics

resolution limits (Å)	30–1.8
R factor (%)[a]	17.3
no. of reflections used	10796
no. of protein atoms	1013
no. of solvent molecules	78
weighted root-mean-square deviations from ideality	
bond length (Å)	0.009
bond angle (Å)	1.786
planarity (trigonal) (Å)	0.013
planarity (other planes) (Å)	0.017
torsion angle (deg)[b]	15.623

[a] R factor $= \sum|F_o - F_c|/\sum|F_o|$. [b] The torsion angles were not restrained during the refinement.

Amine–Borane Complexes as the Reducing Agents. Although the use of sodium borohydride as the reductant resulted in a very high degree of modification, it suffered from the necessity to perform the reaction at high pH leading to an increased number of side reactions and a tendency to denature the protein. This was not a drawback for lysozyme, which is a notoriously robust protein. But once again this type of modification proved to be problematic for myosin subfragment 1. Consequently, a large scale protocol utilizing amine–borane complexes as reducing agents was subsequently developed based on the earlier work of Geoghegan et al. (1981) and Cabacungan et al. (1982). Three different amine–borane complexes were utilized in parallel studies with both lysozyme and myosin subfragment 1 as suggested from these papers: pyridine–borane complex, dimethylamine–borane complex, and triethylamine–borane complex with each showing a decreasing reduction potential in the order as given (Rayment, unpublished results). From these experiments it was deter-

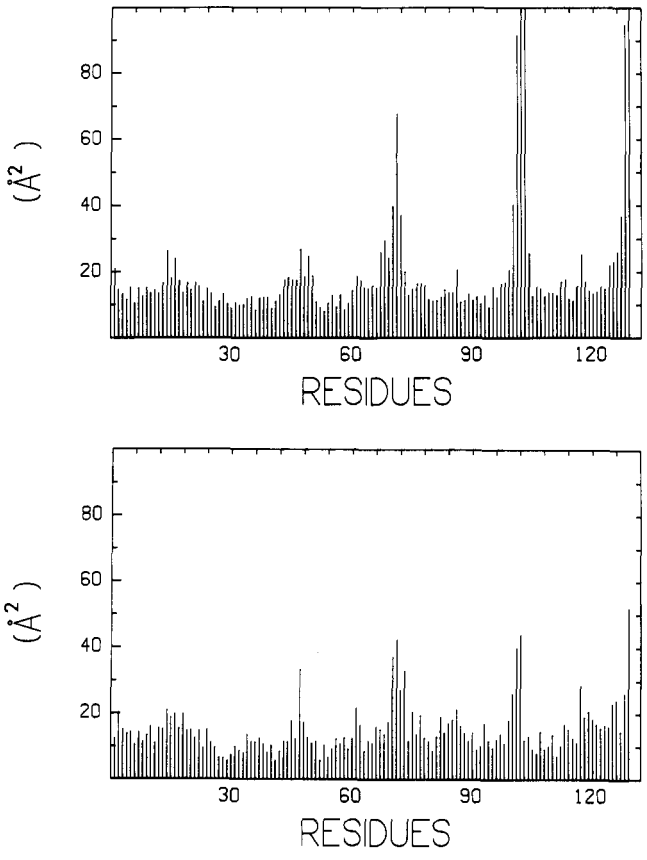

FIGURE 1: Plot of the mean *B* value *versus* amino acid residue for all main chain atoms for both the modified and unmodified forms of lysozyme. (a, top) The only amino acid residues where the polypeptide chain backbone atoms are not well-ordered in the methylated protein are Gly 71, Asp 101, Asn 103, Gly 104, and the C-terminal residues, Arg 128 and Leu 129. (b, bottom) In the unmodified lysozyme, as refined by Wilson et al. (1992), these regions are better ordered.

mined that the pyridine complex tended to denature the protein and was somewhat unstable in solution. Conversely, the triethylamine–borane complex did not result in complete modification. Efforts were thus focused on the dimethylamine–borane complex (DMAB). A variety of reaction protocols were studied with the goal of attaining the highest degree of modification as possible while limiting the residual amounts of formaldehyde. By detecting the rate of consumption of formaldehyde and the extent of modification of the lysine residues, the following protocol evolved that was employed for both myosin subfragment 1 and hen egg white lysozyme (Rayment, unpublished results).

The protein was dialyzed against 200 mM potassium phosphate, pH 7.5. Reductive alkylation was initiated by the addition of 20 μL of 1 M DMAB followed by 40 μL of 1 M formaldehyde per milliliter of protein solution with rapid stirring at 4 °C. The reaction mixture was allowed to stand in the dark for 2 h whereupon the identical addition of DMAB and formaldehyde was repeated. After two more hours a final aliquot of 10 μL of DMAB was added, and the reaction mixture was allowed to stand for an additional 18 h at 4 °C. As before, the reaction was terminated by the addition of 0.5 g of solid ammonium sulfate per milliliter and the precipitate collected by centrifugation. The amino acid analysis of this material is given in Table I. This material crystallized under very similar conditions to those described below for the protein prepared by reduction with sodium borohydride.

Crystallization of Methylated Hen Egg White Lysozyme. Possible crystallization conditions for the modified lysozyme

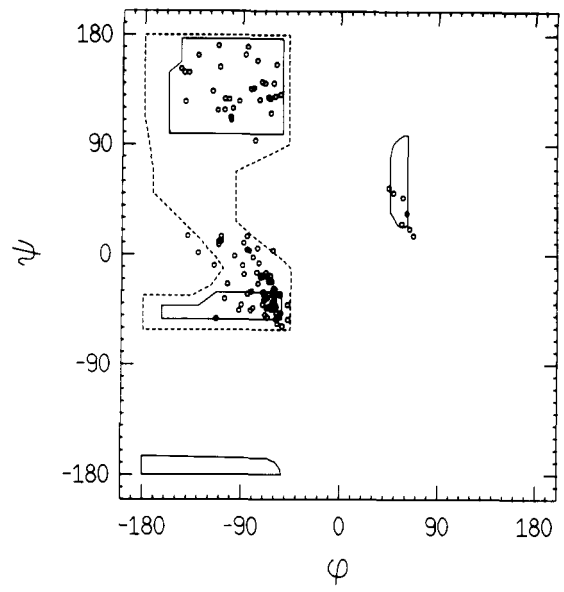

FIGURE 2: Plot of the main chain dihedral angles. A Ramachandran plot of all non-glycinyl main chain dihedral angles for the modified hen egg white lysozyme model is shown. Fully allowed ϕ,ψ values are enclosed by continuous lines; those only partially allowed are enclosed by broken lines.

were surveyed with the hanging drop method of vapor diffusion [for a review, see McPherson (1982)]. Clusters of rod-shaped crystals were first observed growing from $MgSO_4$ solutions buffered with 50 mM Tris, pH 8.0. In order to grow larger crystals, both microdialysis and batch methods of crystallization were employed. Again, the crystals grew as clusters of rods but with some achieving a maximum thickness of 0.2 mm. In order to produce single crystals, the technique of macroseeding was subsequently employed (Thaller et al., 1981, 1985). For seeding, small single crystals were washed in 1.0 M $MgSO_4$ and 50 mM Tris at pH 8.0 and then introduced into small glass vials containing 50–100 μL of a protein solution at 6–7 mg/mL and 1.5 M $MgSO_4$, 50 mM Tris, pH 8.0. Under these conditions, spontaneous nucleation occurred infrequently, and the seeds grew slowly for 2–3 weeks until they reached a thickness of 0.2 mm. At this point, the crystals were transferred for storage to a solution containing 2.2 M $MgSO_4$ and 50 mM Tris at pH 8.0. If the crystals were allowed to grow any larger than approximately 0.2 mm in thickness, they soon became covered with small secondary crystals growing on the surface.

The crystals belonged to the space group $P2_12_12_1$ with unit cell dimensions a = 30.6 Å, b = 56.3 Å and c = 73.2 Å and one molecule in the asymmetric unit. They were stable in the X-ray beam for approximately 70 h.

X-ray Data Collection and Processing. X-ray data were first collected at room temperature to a resolution of 2.4 Å by oscillation photography. The X-ray source was nickel-filtered copper Kα radiation from an Elliot GX20 rotating anode X-ray generator operated at 35 kV and 40 mA with a 200-μm focal cup. The oscillation angle was 2.5° with an exposure time of 4 h per film pack. Because the crystals were 1.5–2.0 mm in length, it was possible to collect a complete X-ray data set from only one crystal. The X-ray films were digitized with an Optronics P1000 film scanner operated with a 50-μm raster and subsequently processed with a set of programs developed by Rossmann (1979). The X-ray data were scaled by the method of Fox and Holmes (1966). The merged X-ray data to 2.4-Å resolution contained 19 165 reflections which reduced to 4844 unique reflections. Three

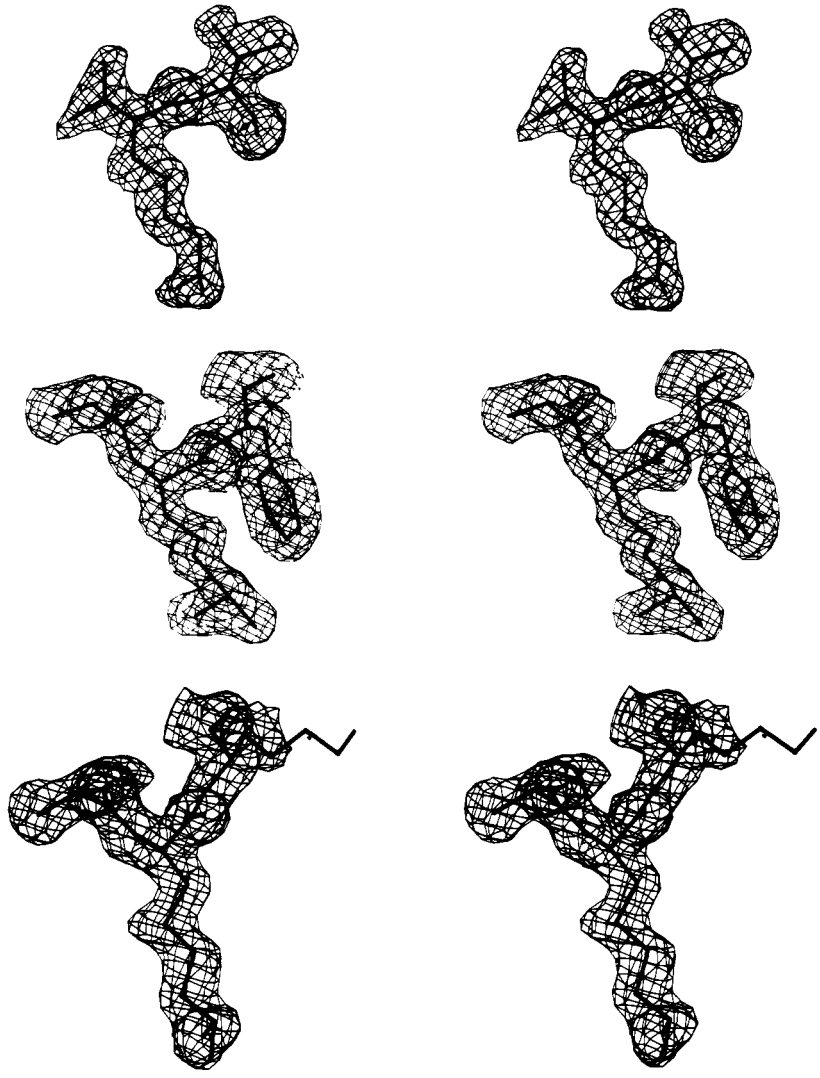

FIGURE 3: Portions of the electron density corresponding to the methylated lysine residues. (a, top) Shown here is the electron density for the N-terminal lysine residue and Val 2. Note that the electron density clearly shows that both the N-terminus and Nζ of the side chain are methylated. (b, middle) The electron density shown here corresponds to Ala 32, Lys 33, and Phe 34. (c, bottom) Shown here is the electron density for amino acid residues Ala 95, Lys 96, and Lys 97.

cycles of postrefinement were performed by the method of Schutt and Winkler (1977). The final R factor for scaling was 5.7% with 93.6% of the reflections having intensities greater than 3σ. These X-ray film data were collected at the University of Arizona and were used to solve the structure of the modified lysozyme by the technique of molecular replacement as described below.

Upon moving to the University of Wisconsin, another X-ray data set was recorded from one crystal to 1.8-Å resolution at 4 °C with a Siemens X1000D area detector system and processed with the XDS data reduction package (Kabsch, 1988). In this case the X-ray source was nickel-filtered copper Kα radiation from a Rigaku RU200 X-ray generator equipped with a 200-μm focal cup and operated at 50 kV and 50 mA. These X-ray data were also internally scaled according to the algorithm of Fox and Holmes (1966). The overall R factor, based on intensity, for the X-ray data set was 2.6%. Relevant X-ray data collection statistics for this native data may be found in Table II. The X-ray data set was 88% complete to 1.8-Å resolution and was used in the least-squares refinement as discussed below.

Molecular Replacement. The three-dimensional structure of the methylated lysozyme was solved by molecular replacement with the software package MERLOT (Fitzgerald, 1988). The refined X-ray coordinates for hen egg white lysozyme as determined in the tetragonal crystal form served as the search model for this study (Diamond, 1974). The cross-rotation function was calculated for different resolution shells of X-ray data. A peak corresponding to the Eulerian angles of α = 52.5°, β = 38.0°, and γ = 155.0° was the largest feature in all calculations, and in the resolution shell from 3.7 to 7.0 Å it appeared at 7.5σ. A solution to the translation problem was determined from an R factor search. At the position, a = 2.5 Å, b = 15.2 Å, and c = 57.1 Å, the R factor dropped from an average value of 70.7% to 49.3%. Further refinement of the rotational and translational parameters yielded a solution at α = 51.8°, β = 37.6°, γ = 155.0°, a = 2.7 Å, b = 15.4 Å, c = 57.3 Å, and an R factor of 40.7%.

Least-Squares Refinement of the Model. The model of the modified lysozyme was refined with the software package TNT using all measured X-ray data from 30.0 to 1.8 Å (Tronrud et al., 1987). Peaks of electron density were considered to be ordered solvent molecules if they were within approximately 3.2 Å of potential hydrogen-bonding groups and if they appeared in both electron density maps calculated with $[2F_o - F_c]$ and $[F_o - F_c]$ coefficients and contoured at 1σ and 3σ, respectively. All positions and temperature factors for the individual protein atoms and the solvent molecules

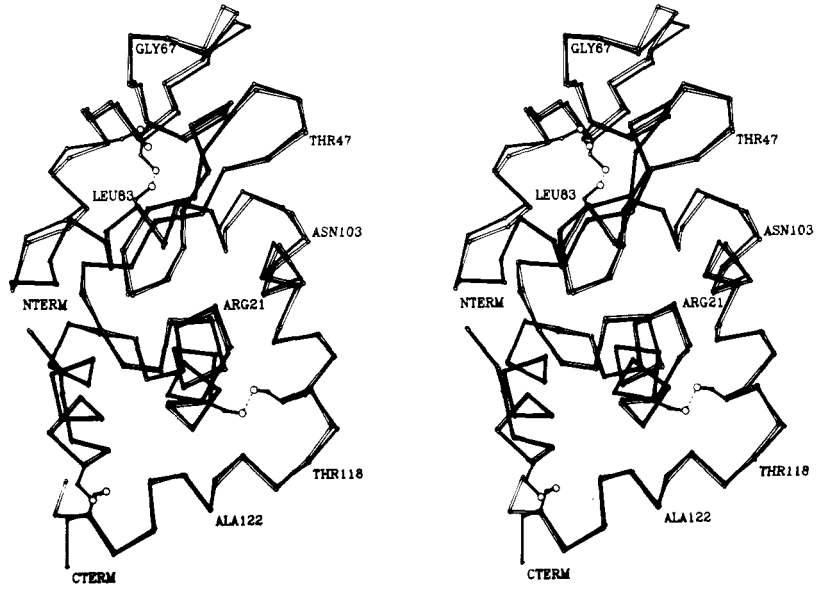

FIGURE 4: Comparison of the α-carbon positions for native and modified forms of hen egg white lysozyme. The superposition shown here was made according to the algorithm of Rossmann and Argos (1975). The native form of the protein is shown in open bonds while the modified molecule is shown in solid bonds. The positions of the disulfide bridges are displayed and various amino acid residues are labeled to aid the reader in following the course of the polypeptide chain. This figure was generated by the software package PLUTO originally written by Sam Motherwell and modified for proteins by Eleanor Dodson and Phil Evans.

Table IV: List of Secondary Structural Elements

amino acid residue no.	secondary structural element
5–14	α-helix
13–16	type I turn
17–20	~type II turn
19–22	~type II turn
20–23	~type I′ turn
25–35	α-helix
43–45	antiparallel β-pleated sheet
46–49	type I turn
50–53	antiparallel β-pleated sheet
54–57	type I turn
58–59	antiparallel β-pleated sheet
60–63	type III turn
69–72	~type II turn
74–77	type I turn
79–82	type III turn
89–100	α-helix
103–106	type II′ turn
109–113	α-helix
115–118	type II turn
119–122	type III turn
122–125	type I turn
124–127	type II turn

Table V: ϕ,ψ Angles for Reverse Turns

amino acid residue no.	type of turn	ϕ_2	ψ_2	ϕ_3	ψ_3
13–16	I	−58.8	−32.3	−88.8	9.2
17–20	~II	−63.6	115.6	67.3	15.4
19–22	~II	−64.2	127.4	55.9	25.4
20–23	~I′	55.9	25.4	91.0	−0.8
46–49	I	−59.1	−29.9	−75.7	5.6
54–57	I	−53.9	−33.3	−109.4	12.0
60–63	III	−78.6	−43.8	−104.8	−37.7
69–72	~II	−69.1	139.6	77.1	29.0
74–77	I	−67.2	−18.2	−89.2	−9.3
79–82	III	−59.7	−28.9	−54.6	−33.4
103–106	II′	63.3	−127.1	−85.8	2.5
115–118	II	−54.5	130.3	94.0	−6.4
119–122	III	−72.7	−19.0	−62.9	−18.6
122–125	I	−71.5	−19.2	−110.1	7.2
124–127	II	−59.9	129.5	79.4	10.1

were refined. The occupancies of the solvent molecules were set to unity. Bond lengths and bond angles for the methylated lysine residues were restrained to typical values observed for carbon–carbon single bonds and for sp^3 hybridized carbon atoms, respectively. Relevant refinement statistics may be found in Table III, and the distribution of the mean main chain temperature factors is given in Figure 1a. As a point of comparison, the mean main chain temperature factors for unmodified hen egg white lysozyme, as refined by Wilson et al. (1992), are given in Figure 1b. Refined X-ray coordinates for the unmodified form were graciously supplied to us by Drs. Keith Wilson and Brian Matthews. X-ray coordinates for the modified lysozyme have been deposited in the *Brookhaven Protein Data Bank* (Bernstein et al., 1977).

DISCUSSION

Chemical modification of proteins has been an often applied technique in structure/function studies. The investigation with hen egg white lysozyme described here was initiated in part to address the issue of the structural consequences associated with such chemical modifications. The molecular structure of hen egg white lysozyme in which the lysine residues have been reductively methylated has now been solved and refined to 1.8-Å resolution and a crystallographic R factor of 17.3%. A ϕ,ψ plot of all non-glycinyl main chain dihedral angles for the refined model is shown in Figure 2. For the most part, the electron density is very well-ordered with the following exceptions. There are several amino acid residues with disordered side chain densities including Arg 14, Arg 45, Arg 68, Arg 73, Arg 112, Arg 114, Gln 121, and Arg 125. Also, the peptide backbone around Gly 71 is weak, and there is a loop, delineated by amino acid residues 101–103, which appears to be disordered. The electron density for Val 99, which leads into this loop, suggests that this residue adopts multiple conformations. The C-terminal amino acid residues, Arg 128 and Leu 129, are likewise not well-ordered. Of the six methylated lysine residues in the modified protein, three have side chain densities that are disordered (Lys 13, Lys 97, and Lys 116). The other three lysines, namely, Lys 1, Lys 33, and Lys 96, are well-ordered as can be seen in the electron density maps presented in Figure 3. Not unexpectedly

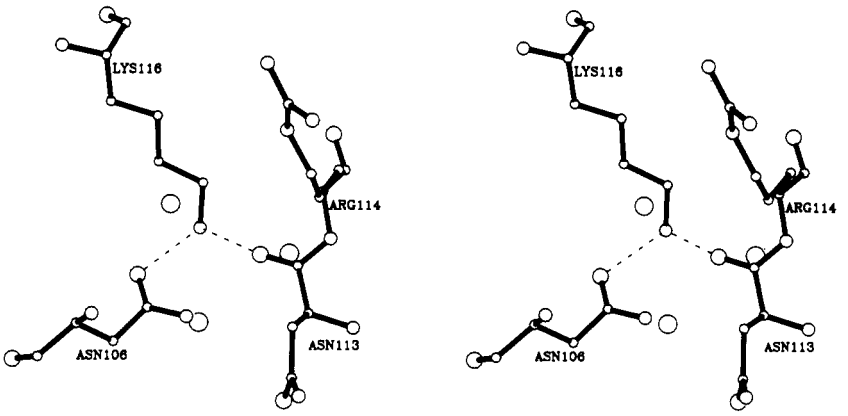

FIGURE 5: Crystalline packing interactions of Lys 116 in the native structure. Shown in stereo is the potential hydrogen-bonding arrangement around Nζ of Lys 116. The open spheres represent the positions of solvent molecules that are within hydrogen-bonding distance of Nζ. Note that Nζ is within 2.9 Å of the carbonyl oxygen of Asn 113, which is located in a symmetry-related molecule in the crystalline lattice. Due to this close contact region between symmetry-related molecules, it is not possible to accommodate a methylated lysine residue in the same conformation. Consequently, the methylated protein crystallized in the space group $P2_12_12_1$ rather than $P4_32_12$ leading to a completely different packing arrangement.

from this type of modification, electron density maps calculated with coefficients of the form $[F_o - F_c]$ revealed that the N-terminus had also been methylated as can be seen in Figure 3a. With respect to the unmodified form of the hen egg white lysozyme, the above-mentioned residues are not disordered in the model refined by Wilson et al. (1992). Whether these differences in order are due to the modification procedure or simply to different crystallization conditions is not known.

A superposition of the modified and unmodified forms of lysozyme is depicted in Figure 4. Hen egg white lysozyme is characterized by four α-helices, three strands of antiparallel β-pleated sheet, five type I turns, one type I′ turn, five type II turns, one type II′ turn, and three type III turns. A list of the amino acid residues participating in these secondary structural elements and the ϕ,ψ angles for the reverse turns may be found in Tables IV and V, respectively.

As can be seen from Figure 4, there are very few structural differences between the modified and unmodified forms of the protein. Specifically, the two proteins differ only in the surface loops defined by amino acid residues 69–72 and 101–103 and the C-terminal amino acid residues 126–129. Excluding these above-mentioned residues, the α-carbon atoms and all atoms of these two proteins superimpose with root-mean-square values of 0.40 and 1.6 Å, respectively. The average temperature factor for the 78 solvent molecules that have been positioned into the electron density is 36.5 Å^2. Of these, 35 adopt similar positions as observed in the native lysozyme structure refined by Wilson et al. (1992).

Crystals of hen egg white lysozyme can be grown easily from approximately 5% sodium chloride, buffered with sodium acetate at pH 4.7 (Blake et al., 1965). Under these conditions, however, the methylated protein did not crystallize. From the structure determination presented here, it is now apparent why the methylated protein did not crystallize in the same space group. In the case of the native protein crystallized in the space group $P4_32_12$, Lys 116, which has low temperature factors, is packed near a symmetry-related molecule such that its side chain nitrogen, Nζ, is 2.9 Å from the carbonyl oxygen of a symmetry-related Asn 113. In addition, as shown in Figure 5, Nζ is 2.8 Å from Oδ1 of Asn 106 in the same molecule. In retrospect, it is not surprising that the methylated protein would not crystallize under the standard conditions employed for lysozyme for the close crystalline contacts between the amino acid residues in this region cannot accommodate the addition of two methyl groups to Nζ of Lys 116. None of the methylated lysine residues are involved in crystalline contacts in the $P2_12_12_1$ unit cell.

Once a protein of interest has been isolated, crystal growth is obviously the first step in any X-ray diffraction analysis. At present, it is often the most troublesome and frustrating part of the structural studies as well. Given the importance that chemical modification has played in the development of biochemistry it is perhaps surprising that this approach had not been previously adopted to solve a crystallization problem. Furthermore, there have been comparatively few structural studies of chemically modified proteins. Those that have been performed have focused on the changes produced in local areas of a protein such as in the active site of an enzyme with the goal of understanding a catalytic mechanism rather than observing the global changes in the molecule as a whole. The investigation presented here clearly demonstrates that careful reductive alkylation of a protein produces only minor structural perturbations. In addition, these changes are similar in magnitude to those observed between different crystal forms of the same protein arising from crystalline packing forces.

There are now numerous examples both in the literature and in our laboratory where the change of a single amino acid residue in a protein results in either a different crystal form or the inability to obtain crystals at all. In principle, this might provide a strategy for growing crystals of proteins that have previously resisted crystallization by changing selected amino acid residues. Unfortunately, it is very difficult to predict which amino acid residue to change by site-directed mutagenesis. Reductive alkylation, on the other hand, offers a more general tool for changing the crystallization properties of a protein. Most importantly, this chemical modification approach produces limited changes in the overall enzymatic properties of most proteins and at least in the case of hen egg white lysozyme results in little three-dimensional structural alterations as well.

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