Pulsed-Alkylation Mass Spectrometry for the Study of Protein Folding and Dynamics: Development and Application to the Study of a Folding/Unfolding Intermediate of Bacterial Luciferase†

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ABSTRACT: A new method employing the classical techniques of chemical modification of proteins and the new technology of mass spectrometry, known as pulsed-alkylation mass spectrometry (PA/MS), has been developed to probe the dynamic structure of folding intermediates and folded complexes of proteins under a variety of conditions. This method is fast and simple, and the results are easily interpreted. PA/MS may provide an alternative to H/D exchange monitored either by NMR or by electrospray ionization mass spectrometry for some experiments; for others, it may provide access to questions not readily answered by available methods. The objective of PA/MS is to determine simultaneously the location and the extent of labeling of functional groups in a protein by measuring the reactivity of cysteines with *N*-ethylmaleimide, within the context of the conformation of the protein under specific conditions. The method can also be applied to chemical modification of other amino acid residues employing any of a vast array of reagents, depending upon the specifics of the protein under investigation. The enormous range of reactivity of the thiol groups of the cysteinyl residues in proteins and the change in reactivity upon denaturation or conformational rearrangement afford a large signal change that can be correlated with changes in accessibility of the thiol group. The information obtained from the correlation of observed thiol reactivity with the local environment of each cysteinyl residue in the structure of the folded protein can be supplemented by results obtained from fluorescence, circular dichroism, or other methods, to develop an understanding of the structure and dynamics of altered conformational states. With bacterial luciferase as a model system, we have applied PA/MS to investigate the structural differences between the native heterodimeric enzyme and a folding intermediate that is well-populated in 2 M urea. The thiol residues at positions 307, 324, and 325 of the α subunit were much more reactive with *N*-ethylmaleimide in the presence of 2 M urea than in the native enzyme, suggesting that the C-terminal region of the α subunit was less tightly packed in the folding intermediate. The apparent unfolding of the C-terminal region of the α subunit of the αβ structure in 2 M urea appears to mimic the unfolding of the C-terminal domain of the free α subunit, also in 2 M urea, described by Noland, B. W., Dangott, L. J., and Baldwin, T. O. (1999) *Biochemistry 38*, 16136−16145. The approach described here should be applicable to a wide array of problems that have in common the need to determine the locations of conformational changes in proteins. Application of PA/MS to the investigation of the relative thermodynamic stability of the coordination complexes of zinc within each of the six zinc-finger domains of MRE-binding transcription factor-1 (Zn_6 MTF-zf) in its free and DNA-bound forms is presented in the companion paper in this issue [Apuy, J. L., Chen, X., Russell, D. H., Baldwin, T. O., and Giedroc, D. P. (2001) *Biochemistry 40*, 15164−15175].

The requirement to fold to the biologically active three-dimensional conformation is a critical function shared by all proteins. The amino acid sequence of a protein contains, at the most fundamental level, the information that dictates the structure (*1*). In addition to the structure, the amino acid sequence must also dictate the conformational flexibility of the polypeptide. It is well appreciated that the biological activity of a protein depends on both the structure and the dynamics of the folded polypeptide. To truly understand the function of a protein, it is necessary to know in detail its three-dimensional structure and how the conformation changes during folding and function. Although the number of possible interactions of the component residues of a folding polypeptide is huge, many proteins do fold spontaneously into their native conformations under physiological conditions. Achieve-

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ment of the native structure on a time scale of milliseconds to seconds is possible only if the protein samples a very small fraction of all possible conformations during folding (*2*). The ordered sets of conformations leading to the native state collectively define the folding pathway.

Transient intermediates that define protein folding pathways differ in population size depending on the height of the potential energy barrier that has to be overcome for that intermediate to proceed through the folding pathway relative to the height of barriers that precede the specific intermediate. Stable intermediates in folding reactions are identified by monitoring folding and unfolding of the protein, usually by spectroscopic techniques such as circular dichroism or fluorescence. An understanding of these intermediate structures leading to the folded state is critical for understanding the mechanism of protein folding. Several techniques have been developed to study such transient structures. One approach is H/D exchange-2D NMR, which monitors the disappearance of specific ^{1}H NMR peaks due to deuterium replacement of accessible and exchangeable protons, permitting inference of which regions of the protein are exposed to the solvent (*3*). NMR methods provide information about the population average exchange at specific sites in the protein but not the extent of exchange in these sites within individual molecules. The method is also limited to smaller proteins and to those proteins for which the proton NMR assignments have been completed. For larger proteins, such as bacterial luciferase, that form multisubunit complexes, the size of the protein and number of interactions complicate the NMR spectra such that peak deconvolution is difficult or impossible. Another approach is the use of H/D exchange-electrospray ionization mass spectrometry (H/D-ESI-MS)[1] to determine regions in the protein that are susceptible to exchange as determined by a shift to a higher mass-to-charge (*m*/*z*) ratio of the proteolytic fragments of the protein that undergo exchange (*4*). However, this method does not provide residue-specific information. Also, it is difficult to quantitate unambiguously the extent of H/D exchange because of the dynamic equilibrium between hydrogen and deuterium that may result in a redistribution of the label in the peptides during sample preparation for mass spectrometry. The only way to determine the extent of labeling at a specific amino acid residue is by using both mass spectrometry and NMR to gather complementary information about the average population distribution and average proton occupancy at individual sites (*5*).

Early applications of chemical modification were indeed elegant and yielded clear evidence of activity-related changes in conformation. One such study showed that the cysteinyl residue at β93 of human hemoglobin was reactive with *N*-ethylmaleimide when the hemoglobin was in the oxygenated form, but was unreactive in deoxyhemoglobin (*6*). Furthermore, this modification caused a dramatic reduction in the Bohr effect, the pH dependence of oxygen affinity. The reactivity of the cysteine at β93 in oxyhemoglobin was the clue needed by Perutz to use mercurials to obtain the required phasing of his X-ray diffraction data to solve the hemoglobin structure (*7*). From the crystal structure, it became clear that the change in reactivity was the result of formation of a salt bridge between the C-terminal histidine of deoxyhemoglobin and the aspartate at β92, a structural rearrangement that blocked access of NEM to the thiol at β93 (*6*), suggesting the structural basis for at least part of the Bohr effect.

Using a family of *N*-alkylmaleimides, Anderson and co-workers probed the hydrophobicity of the environment surrounding specific residues (*8*, *9*). The intrinsic reactivity of the maleimides is relatively insensitive to the nature of the N-alkyl substituent, while the rates of reaction with certain protein thiols are dramatically altered by the nature of the N-alkyl substituent. Increasing the chain length of the alkyl substituent in maleimides enhances the rate of inactivation of papain and D-amino acid oxidase, apparently through hydrophobic interactions with a nonpolar environment on the enzyme prior to covalent reaction (*8*, *9*). Similar studies with bacterial luciferase showed that the environment surrounding the reactive thiol at α106 is hydrophobic (*10*). Kenyon and co-workers (*11*) used methyl methanethiolsulfonate to introduce a small modification (-S-CH_3) onto the reactive thiol of rabbit muscle creatine kinase, finding residual activity for the modified enzyme, demonstrating the nonessentiality of the sulfhydryl for catalysis. Incorporation of the larger *S*-ethyl or *S*-propyl groups reduced the residual enzymatic activity further. Wallenfels and Eisele (*12*) studied the stereospecificity of reaction of the D- and L- isomers of α-iodopropionic acid with papain, showing that the L-isomer reacts faster than the D-isomer with the reactive sulfhydryl group in papain. They attributed the difference in the reaction rate to the better orientation of the L-isomer of α-iodopropionic acid in the modifying site (*12*).

On the basis of these and numerous other contributions to the protein chemistry literature, it is clear that the rate of reaction of a specific residue with a modifying reagent is influenced by the reactivity of the residue (which depends on the pH and the pK_a of the functional group) and also by the accessibility of the residue to the reagent and the orientation of the functional group relative to the reagent. Clearly, changes in protein structure result in large changes in reactivity of specific functional groups, so changes in the rate of reaction have been used for many years to probe conformational changes in proteins. Recent developments in biological mass spectrometry promise to enhance the value of chemical modification for the study of protein structure and folding.

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been widely used in many areas to solve biological problems since its introduction in 1987 (*13*). Protein identification by peptide mass mapping strategies has especially benefited from the application of MALDI-TOF-MS (*14*−*18*). In addition to protein identification, MALDI-TOF-MS is used to study conformational changes of proteins by monitoring chemical modification of specific amino acid residues (*19*−*21*). There are several reasons why MALDI-TOF-MS is preferred over other mass spectrometric techniques. Sample preparation is

[1] Abbreviations: pulsed-alkylation mass spectrometry, PA/MS; hydrogen−deuterium exchange, H/D exchange; nuclear magnetic resonance, NMR; matrix assisted laser desorption ionization time-of-flight mass spectrometry, MALDI-TOF-MS; delayed extraction, DE; endoproteinase Asp-N, AspN; *N*-methylmaleimide, NMM; *N*-ethylmaleimide, NEM or H_5-NEM; *N*,*n*-propylmaleimide, NPM; *N*,*n*-octylmaleimide, NOM; β-mercaptoethanol, βME; deuterated *N*-ethylmaleimide, d_5-NEM; trifluoroacetic acid, TFA; ethylenediamine tetraacetate, EDTA; dalton, Da; atomic mass units, amu; nanometer, nm; signal-to-noise ratio, S/N; mass-to-charge ratio, *m*/*z*.

straightforward because MALDI is relatively tolerant to chemical denaturants and chemical modifiers. Data acquisition/analysis requires less time because MALDI mass spectra are rather simple. Mostly singly charged ions are observed in the MALDI mass spectra, and more information can be extracted from a single mass spectrum. Most importantly, MALDI-TOF-MS can achieve a very high level of mass accuracy (<10 ppm) at low mass range (0.5∼5 kDa) with the aid of delayed extraction and reflectron technology (*22*−*27*). As a result of high mass-accuracy mass measurements, proteins can be unambiguously identified with fewer digest fragments in protein database searching (*28*−*31*). Furthermore, accurate masses of digest fragments can provide accurate information about the change in amino acid composition due to mutation, chemical, or posttranslational modification. High mass accuracy MALDI-TOF mass measurement has been used to characterize the substituent group on the flavin of nitroalkane oxidase (*32*, *33*). In the present study, proteolytic digest fragments of bacterial luciferase were first identified, and then the extent of chemical modification in both intact protein and digestion fragments of bacterial luciferase was monitored by MALDI-TOF-MS.

Bacterial luciferase from *Vibrio harveyi*, a heterodimeric aldehyde monooxygenase (ca. 76 kDa) that catalyzes a bioluminescent reaction of $FMNH_2$, aliphatic aldehyde, and O_2, provides an excellent model system for the study of subunit folding and assembly of large proteins (*34*−*39*). The proposed folding pathway, as determined by refolding studies monitored by enzyme activity assay, fluorescence emission, and circular dichroism, reveals that the unfolded α and *β* subunits fold into dimerization competent species that then assemble to form the heterodimeric intermediate. This intermediate complex isomerizes to form the active enzyme. An intermediate that is well-populated at equilibrium in 2 M urea has been identified spectroscopically, but minimal structural information is available (*37*). The high-resolution structure of the α*β* heterodimer (*40*) and of the β_2 homodimer (*41*) provides the location and orientation of almost all the side-chains of the residues in the folded protein.

V. harveyi luciferase has a total of 14 cysteinyl residues (*42*, *43*) widely distributed throughout the structure (Figures 1 and 2) that may serve as useful probes to detect localized changes in protein conformation. The purpose of this paper is to describe the PA/MS method and to report preliminary studies of its application to the study of a folding intermediate of bacterial luciferase that is well-populated in 2 M urea (*37*, *38*).

MATERIALS AND METHODS

Materials. *N*-methyl-, *N*-ethyl-, and *N*,*n*-propylmaleimide, dithiothreitol (DTT), EDTA, ultrapure urea, NaH_2PO_4, and K_2HPO_4 were products of ICN. *N*,*n*-octylmaleimide was custom-synthesized by Pfaltz & Bauer Inc. Flavin mononucleotide, *n*-decanal, Sephadex G-25, *β*-mercaptoethanol, TPCK-treated trypsin, chymotrypsin, AspN, bradykinin, horse heart myoglobin, and ferulic acid were from Sigma. α-Cyano-4-hydroxycinnamic acid was supplied by Aldrich. Methanol and acetonitrile (HPLC-grade) were from EM Science. C4 and C18 microtip columns were purchased from Amika Corp. The *N*-ethylmaleimide-d_5 (d_5-NEM) was prepared for us by Medical Isotopes Inc. For storage, the

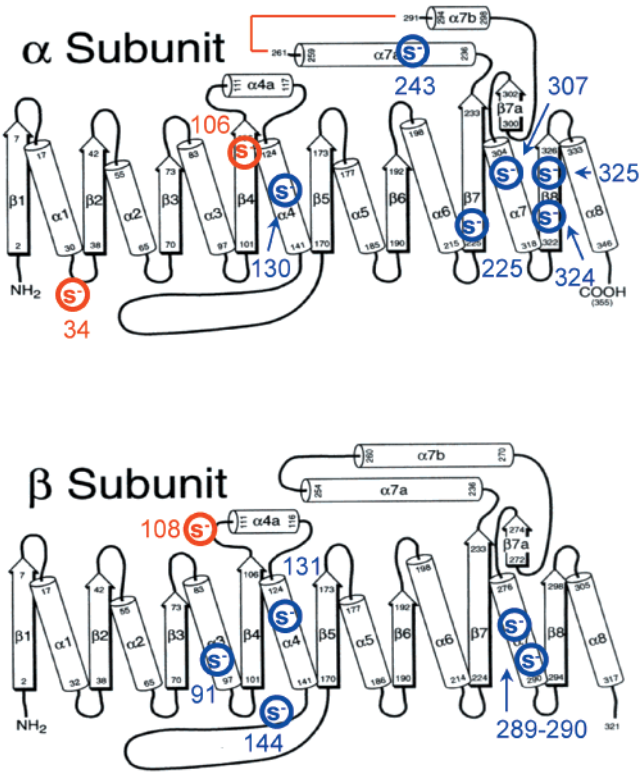

FIGURE 1: The location of cysteinyl residues in the secondary structure of the α and *β* subunits of *V. harveyi* luciferase. Cysteinyl residues at locations designated by residue numbers are represented by circled S^- indicators superimposed on the secondary structural representation of the subunits. Surface-accessible cysteinyl residues are shown in red, and nonsurface residues are shown in blue. The red line in the α subunit represents the unresolved mobile loop region. Arrows and cylinders are representative of *β*-strands and α-helices, respectively. In each subunit, strand *β*8 wraps around and hydrogen bonds to *β*1 to form a closed *β*-barrel core surrounded by intervening α-helices.

maleimides and *β*-mercaptoethanol were placed in a desiccator over a commercial desiccant and stored at −20 °C. Stock solutions of the maleimides at 0.1 M were prepared in 50% acetonitrile solution; 1.4 M *β*-mercaptoethanol was prepared in Milli-Q water. All *N*-alkylmaleimide labeling experiments were performed in 50 mM phosphate buffer, pH 7, at 18 °C.

Luciferase Purification and Assay. Escherichia coli (LE392) cells carrying the *V. harveyi luxAB* genes on a pUC9-derived plasmid, pLAV1, were grown, and the luciferase was purified as previously described (*44*). Enzyme concentration was determined by absorbance at 280 nm, using an extinction coefficient of 1.13 $(mg/mL)^{-1}$ cm^{-1} (*45*). The enzyme activity was assayed at room temperature using a photomultiplier-photometer to detect light emission from a reaction initiated by rapid injection of $FMNH_2$ into a vial containing luciferase in buffer, oxygen, and *n*-decyl aldehyde (*44*, *46*).

Preparation of Luciferase to Study Conformational Stability by Sensitivity to Limited Proteolysis. A sample of *V. harveyi* luciferase at 2 mg/mL in 50 *μ*L of 50 mM phosphate buffer solution, pH 7.0, was allowed to react with 2.0 *μ*L of 0.1 M *N*-*n*-octylmaleimide for 5 min. The reaction was quenched by addition of 2 *μ*L of 1.0 M *β*-mercaptoethanol, and the sample was passed through a Sephadex G-25 desalting column with a capacity of 50−100 *μ*L to remove excess quenching agent and reaction products. The sample

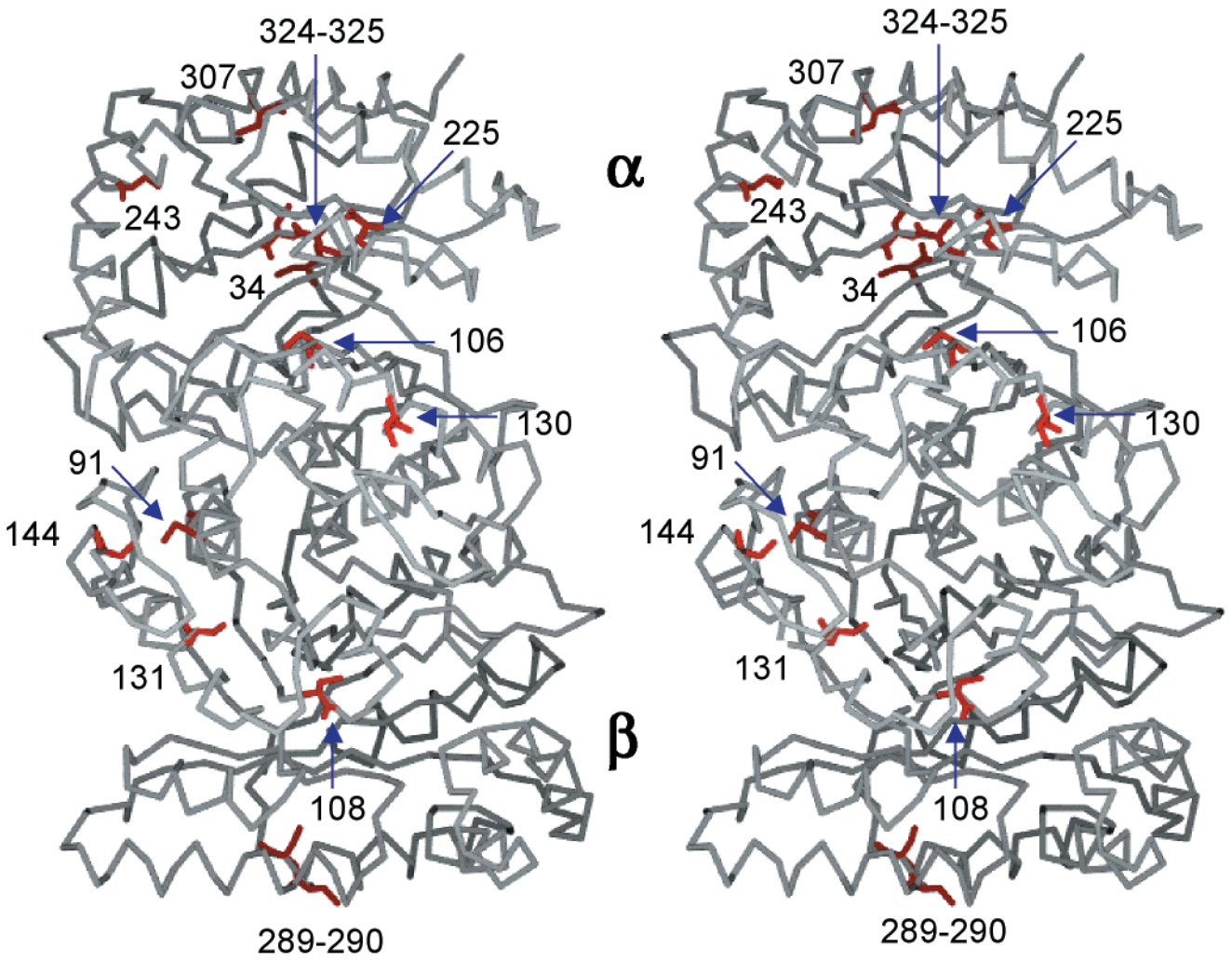

FIGURE 2: The distribution of cysteinyl residues in the quaternary structure of the $\alpha\beta$ heterodimeric luciferase from *V. harveyi*. The red residues are the cysteinyl residues at positions designated by residue number. The 14 cysteines are well-distributed throughout the three-dimensional structure of the protein, providing useful probes to detect localized changes in protein conformation.

was then digested with chymotrypsin at a protein-to-protease mass ratio of 15. At time intervals (30 s, 1, 2, 5, 10 min), aliquots were removed for acid-induced quenching of digestion using trifluoroacetic acid. A 2 μL aliquot of each sample was then diluted to a final concentration of 0.1 mg/mL in 40 μL of a 1.5 mg/mL solution of ferulic acid in 25% aqueous methanol. These samples were then deposited as a series of 500 nL spots on top of an air-dried layer of matrix made by spotting 5 μL of a 30 mg/mL ferulic acid solution in pure methanol. Samples were subsequently air-dried before mass spectral analyses were performed in the mass spectrometer adjusted to linear mode settings suitable for high mass determination.

Sample Preparation for Identification of Cysteine-Containing Peptides. Theoretical proteolytic digests of *V. harveyi* luciferase subunits were tabulated using MS-DIGEST available at the www.prospector.ucsf.edu website. To locate single cysteine-containing proteolytic peptides in the mass spectrum, the output from the mass spectral analysis was scanned for peptide fragments of the expected mass. To locate modified peptides, the expected masses were increased by the mass of the modifying group and the output again scanned. To do a proteolytic digest, a 1 mg/mL protein sample in 50 μL of 50 mM phosphate buffer solution, pH 7.0, was mixed with 4 μL of a 1 mg/mL solution of either trypsin or AspN, and the digestion was allowed to proceed for 4 h (trypsin) or 12 h (AspN). Following digestion, an equal aliquot was allowed to react with 140× molar excess *N*-ethylmaleimide (NEM) relative to protein (10× on a per thiol basis) at 18 °C for 1 h before adding a 10× molar excess of dithiothreitol relative to NEM. A 4 μL aliquot of each sample was then diluted to a final concentration of 0.1 mg/mL in 40 μL of a 1.5 mg/mL α-cyano-4-hydroxycinnamic acid solution in 25% aqueous methanol. These samples were deposited as a series of 500 nL spots on top of an air-dried layer of matrix made by spotting 5 μL of a 30 mg/mL solution of α-cyano-4-hydroxycinnamic acid in pure methanol. Samples were subsequently air-dried before mass spectral analyses were performed in the mass spectrometer adjusted to reflectron mode settings suitable for low mass determination (500−2500 Da). For higher mass determination (2000−5000 Da), the same procedure was followed, substituting ferulic acid rather than α-cyano-4-hydroxycinnamic acid as matrix and adjusting the mass spectrometer to linear mode settings.

Preparation of Samples to Evaluate Strategies to Quantitate the Extent of Maleimide Modification of Cysteine-Containing Peptides. A 2 mg/mL sample of *V. harveyi* luciferase in 250 μL of 50 mM phosphate buffer, pH 7.0, was digested with trypsin for 4 h as described above. The sample was then divided into five equal aliquots. A control

aliquot was allowed to remain unmodified, while experimental aliquots were allowed to react with *N*-ethylmaleimide (NEM), *N*-methylmaleimide (NMM), *N*-*n*-propylmaleimide (NPM), or d_5-NEM at 18 °C for 1 h. All reactions were then quenched with β-mercaptoethanol. To evaluate the effects, if any, of the alkyl chainlength on ion yield upon mass spectral analyses, equimolar mixtures of the NMM-, NEM-, and NPM-modified samples were prepared. In addition, equimolar mixtures of NMM- and NEM-modified samples and of NEM- and NPM-modified samples were prepared. H_5-NEM- and d_5-NEM-modified samples were also mixed in H_5:d_5 concentration ratios ranging from 1:9 to 9:1. These samples were then subjected to mass spectral analyses using the procedure cited above.

Pulsed-Alkylation Protocol for Investigating the Reactivity of Cysteinyl Residues under Native Conditions. A 3 mg/mL sample of *V. harveyi* luciferase in 100 μL of 50 mM phosphate buffer, pH 7.0, was used for the experiment. The reaction was initiated by addition of 2 μL of a 1 M d_5-NEM solution while vortexing and allowed to proceed for 3 min. After 3 min, 20 μL of a 1 M H_5-NEM solution was added to completely modify all remaining cysteinyl residues.

Pulsed-Alkylation Protocol for Investigating Local Conformational Changes in the Presence of Denaturants. A 25 μg/mL sample of *V. harveyi* luciferase was incubated in 5 mL of 2 M urea, 50 mM phosphate buffer, pH 7.0, at 18 °C for 15 h. Following incubation, 23 μL of a 0.01 M NEM solution was added to the 5 mL sample and allowed to react for 5 s while the sample was being vortexed. After 5 s, 23 μL of a 0.1 M β-mercaptoethanol solution was added to quench the reaction. The sample was then subjected to mass spectral analyses using the procedure outlined above.

Pulsed-Alkylation Mass Spectrometry Cleanup Steps. Urea and excess reagents were removed from samples that had been alkylated in the presence of urea by introduction of the samples to an acid-washed Amika C4 microtip column. The columns were washed with 2 mL of 0.1% TFA solution, and the protein was eluted with 500 μL of 0.1% TFA−acetonitrile solution. Samples were subjected to two cycles of concentration and dilution with deionized/distilled water in a heated SpeedVac to remove residual acetonitrile. Samples in a final volume of 50 μL were then neutralized with 3−10 μL of 0.1 M ammonium bicarbonate if tryptic digestion were to follow. Samples were then subjected to mass spectral analyses using the procedure described above.

MALDI Spectra. The MALDI-DE-TOF mass spectra of the samples were acquired using a Perseptive Biosystem Voyager Elite XL TOF mass spectrometer equipped with a pulsed nitrogen laser emitting at 337 nm (Laser Science Inc.). All spectra were acquired in the positive ion mode using 25 kV acceleration voltage. Each spectrum is the average of 100 laser pulses. The spectra for the intact subunits and larger tryptic peptides of bacterial luciferase were acquired with 525 ns delayed extraction, and the low mass gate was set to exclude all ions below *m*/*z* 800. The grid and the guide wire voltages were operated at 93 and 0.05% of the acceleration voltage, respectively. The spectra for the smaller tryptic fragments were acquired with 200 ns delayed extraction in reflectron mode. The grid and the guide wire voltages were operated at 70 and 0.05% of the acceleration voltage, respectively. Horse heart myoglobin (16 951.5 Da) and bradykinin (1060.5 Da) were used for external calibrations of the mass spectrometer prior to mass analyses of intact subunits and tryptic peptides, respectively.

RESULTS

Limited Chymotryptic Digest to Probe Conformational Stability. Application of MALDI-TOF-MS to analyze a limited chymotryptic digest of the *V. harveyi* $\alpha\beta$ heterodimer, earlier investigated by Rausch using SDS gel electrophoresis (*47*), revealed high-resolution information about initial cleavage events in both subunits. Under native conditions, sequential cleavage within discrete regions of the enzyme was observed. The most protease-sensitive site was the disordered loop region, residues 261−290 in the α subunit (*40, 42*). Complementary peptide fragments resulting from amide bond hydrolysis between residues 270−271 and 280−281 were detected simultaneously (Figure 3). Further digestion produced secondary complementary peptide fragments that corresponded to the hydrolysis of amide bonds between residues 117−118 in the interfacial pocket region of the α subunit and subsequently between residues 117−118 in the interfacial pocket region of the β subunit. When protein was partially labeled with NOM (3 in α, 1 in β), the same sequential cleavage events were observed.

Qualitative Analysis of Results of PA/MS. For mass spectrometric analysis, it was necessary first to identify all the unmodified and alkylated forms of the intact subunits and their cysteine-containing protease-derived peptides in the mass spectra. Figure 4 displays a complete tryptic digest of luciferase. There were 9 peptides with 1 cysteine each, 1 peptide (from β) that contains 2 cysteines, and 1 peptide (from α) that contains 3 cysteines (Table 1). The cysteinyl residues in the peptides containing 2 and 3 residues reside in the C-terminal region of the β and the α subunit, respectively, and thus can report as a group conformational rearrangement in that region of each subunit. Because of the absence of a tryptic cleavage site in the peptide that contains the three cysteinyl residues in α, we resorted to AspN digestion of the heterodimer to resolve the NEM-modification of αC307 from those of αC324−325 (Figure 5, Table 2).

Quantitative Analysis of Results of PA/MS. To accurately quantitate the relative ion abundance between unmodified and alkylated peptides, there must be a linear correlation between the true concentration ratio and the ion abundance ratio of the two forms of each peptide. However, measurement of the peak area ratios for these peptides did not yield a linear relationship. All the cysteine-containing peptides showed an increase in the ion abundance as a result of labeling (Figure 6A). As a consequence, it was obvious that comparisons of peak areas of modified and unmodified peptides would not yield reliable quantitation.

As an alternative approach, we developed a double-label strategy using N-alkylmaleimides of different N-alkyl chainlength. This approach was tested by using equimolar mixtures of trypsin-digested bacterial luciferase that were individually modified using N-alkylmaleimides that differ chemically from NEM by one methylene group. The effects of the *N*-methyl, *N*-ethyl, and *N*,*n*-propyl substituents on relative ion yield for two peptides are shown in Figure 7. From these comparisons and others not shown, it was obvious that the N-alkyl chainlength can have a strong effect on ion abundance, an observation that is at this time unexplained.

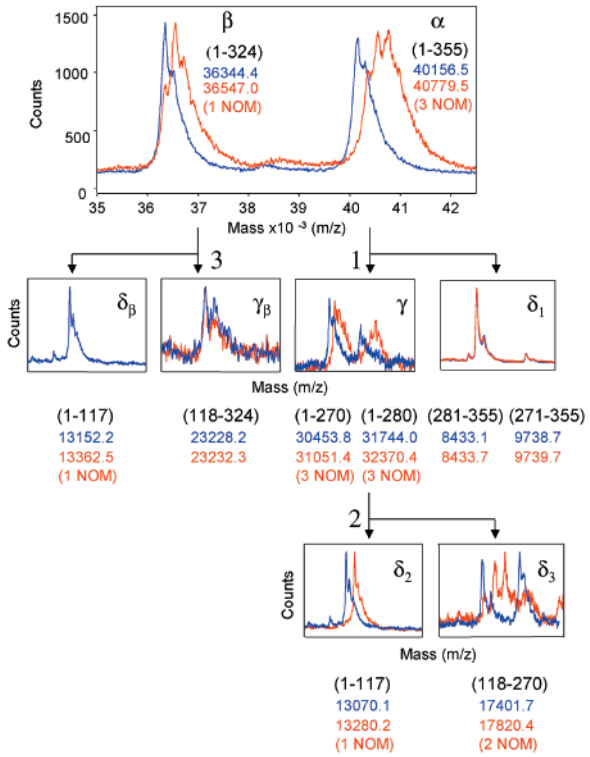

FIGURE 3: Superimposed MALDI-DE-TOF mass spectra of unmodified (blue trace) and partially *N*,*n*-octylmaleimide (NOM) modified (red trace) forms of *V. harveyi* luciferase subunits and of the initial peptide fragments generated by limited chymotrypsin digestion. The structure on the high mass side of the unmodified subunit peaks is due in part to isotopic heterogeneity and possibly to sodium and/or potassium ion adducts. The mass spectra of the intact subunits show that the α and the *β* subunits are partially modified with three and one NOM, respectively. The α subunit spectrum showed three species, a singly modified, a doubly modified, and a triply modified form, but essential no unmodified subunit. The *β* subunit, on the other hand, showed a significant amount of unmodified protein, as well as singly modified subunit. The number of modifications was determined by the difference in mass between the unmodified and modified forms of the subunit divided by the mass of NOM (209 Da). Identical peptide fragments of the unmodified (in blue) and partially NOM-modified (in red) forms of *V. harveyi* luciferase were generated through limited chymotrypsin digestion. Two sets of complementary peptide fragments (γ and δ_1) were simultaneously generated through hydrolysis of peptide bonds at amino acid positions 270 and 280 in the disordered region of the α subunit (panel 1). Subsequent hydrolysis of γ at α-117 divides γ into δ_2 and δ_3 (panel 2), before hydrolysis of the peptide bond at *β*-117 fragments *β* into γ_β and δ_β (panel 3). Note in panel 2 that the unmodified (blue trace) δ_3 fragment is the lower mass of the two peaks. Upon modification, the δ_3 fragment showed a small amount of unmodified peptide, as well as singly and doubly modified product. The identity of the lower mass peak was not determined. Comparison of the chymotryptic fragmentation patterns of the modified and unmodified proteins shows that there were no significant conformational perturbations on the overall structure of the enzyme due to limited modification.

As a result of the unexpected effects of the modifying reagent on quantitation of the mass spectra, we decided to use the alkyl deuteriomaleimide, d_5-*N*-ethylmaleimide (d_5-NEM). Using deuteriomaleimide and proteomaleimide in a mixed-label experiment, we found no effect of the isotopes on ion abundance of the cysteine-containing peptides (Figure 8). Furthermore, the measured ratio of peak areas versus experimentally established molar ratios yielded a straight line of unit slope (Figure 9). By this method, we determined the

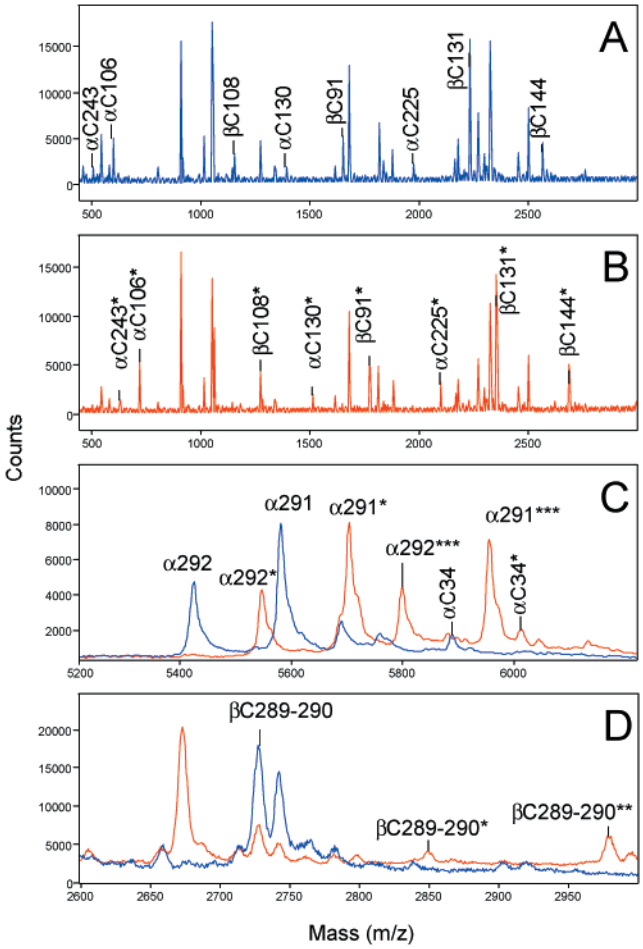

FIGURE 4: MALDI-DE-TOF mass spectra of unmodified and *N*-ethylmaleimide (NEM) modified forms of all cysteine-containing tryptic peptides of *V. harveyi* luciferase. Eight unmodified, single cysteine-containing tryptic peptides were identified by their mass-to-charge (*m*/*z*) ratios (A) and their identities were confirmed by an observed mass shift of 125 Da in their mass to charge (*m*/*z*) ratio upon exposure to NEM (B). Superimposed mass spectra (C) identify two tryptic peptides that contain the same three cysteinyl residues (307, 324, and 325), but differ by an argininyl residue (αR291) at the N-terminus. These two peptides with N-termini of αR291 and αI292 (designated α291 and α292, respectively) were generated by incomplete cleavage of the Arg−Arg dipeptide at α290−291. The spectra in panel C also show a single cysteine-containing tryptic peptide and its modified form. Superimposed mass spectra (D) identify a two cysteine-containing tryptic peptide and its NEM-modified forms. Amino acid sequence information and the mass of each peak are provided in Table 1. The number of asterisks (*) next to the residue number indicates the number of NEM modifications in each tryptic peptide.

order of reactivity of the cysteinyl residues in the heterodimer under native conditions as follows: αC106 > *β*C108 > αC130 > *β*C131 > αC243 > *β*C91 > αC225 > *β*C144 (data not shown).

Probing the Structure of the αβ Heterodimeric Intermediate in 2 M Urea. The previous studies of Noland et al. (*39*) of the free α subunit and its 2 M urea folded intermediate have suggested that the C-terminal domain of the protein undergoes partial unfolding in 2 M urea. We subjected the *αβ* heterodimer to identical denaturing conditions to investigate the possibility of partial unfolding of the subunits in the heterodimer. Mass spectral analyses of a 5-s pulse-labeled sample in 2 M urea showed a distribution of one and three NEM modifications of the intact α subunit, while none were

Table 1: Amino Acid Sequences and Masses of Unmodified and Differently NEM-Labeled Tryptic Peptides of Bacterial Luciferase[a]

cysteinyl residue	amino acid sequence of *V. harveyi* luciferase	mass of NEM-labeled peptides (Da)							
		unmodified		1 NEM		2 NEM		3 NEM	
		obs mass	calc mass	obs mass	calc mass	obs mass	calc mass	obs mass	calc mass
αC243	[241]DICR	506.22	506.24	631.29	631.28				
αC106	[103]FGICR	595.29	595.30	720.33	720.34				
*β*C108	[101]FAFGFSDCEK	1150.44	1150.49	1275.49	1275.54				
αC130	[126]ALMDCWYDLMK	1388.55	1388.61	1513.59	1513.66				
*β*C91	[086]VAEEACLLDQMSEGR	1650.69	1650.75	1775.74	1775.80				
αC225	[222]IDHCLSYITSVDHDSNR	1974.83	1974.90	2099.88	2099.94				
*β*C131	[116]FFNRPTDSQFQLFSECHK	2230.98	2231.03	2356.01	2356.08				
*β*C144	[134]IINDAFTTGYCHPNNDFYSFPK	2564.09	2564.16	2689.13	2689.21				
[291]αC307,324−325	[291]R/[292]IDYSYEINPVGTPEE	*5581.67*	*5581.24*	*5706.30*	*5706.29*		*5831.33*	*5956.07*	*5956.38*
[292]αC307,324−325	CIAIIQQDIDATGIDNICCG	*5425.67*	*5425.06*	*5550.70*	*5550.11*		*5675.15*	*5800.87*	*5800.20*
	FEANGSEEEIIASMK								
αC34	[30]ASEGCGFDTVWLLEHHFT	*5887.62*	*5887.74*	*6012.31*	*6012.78*				
	EFGLLGNPYVAAAHLLGA								
	TETLNVGTAAIVLPTAHPVR								
*β*C289−290	[285]VAIECCGAADLLMSFESMEDKA	*2727.61*	*2727.18*	*2852.90*	*2852.22*	*2977.22*	*2977.27*		
	QQR								

[a] Masses of molecular ion peaks are in regular fonts and masses of average peaks are in italicized font. Subunits are designated by Greek letters (α or *β*) before residues.

Table 2: Amino Acid Sequences and Masses of Unmodified and Differently NEM-Labeled AspN Peptides of Bacterial Luciferase[a]

cysteinyl residue	amino acid sequence	mass of NEM-labeled peptides (Da)							
		unmodified		1 NEM		2 NEM		3 NEM	
		obs mass	calc mass	obs mass	calc mass	obs mass	calc mass	obs mass	calc mass
αC307	[293]DYSYEINPVGTPEECIAIIQQ	*2454.20*	*2454.73*	*2579.56*	*2579.78*				
αC324−325	[321]DNICCGFEANGSEEEIIASMKLFQS	*2879.52*	*2879.23*	*3004.62*	*3004.28*	*3129.93*	*3129.36*		

[a] Masses of molecular ion peaks are in regular fonts, and masses of average peaks are in italicized font. Subunits are designated by Greek letters (α or *β*) before residues.

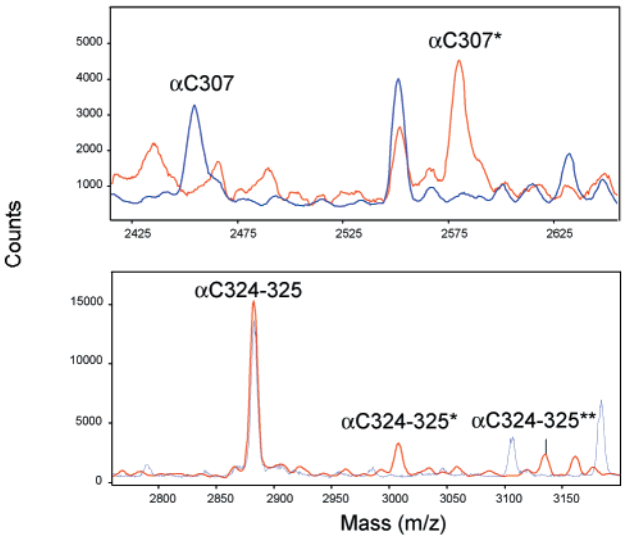

FIGURE 5: MALDI-DE-TOF mass spectra of two cysteine-containing peptides generated by complete AspN digestion and their NEM-modified forms. AspN protease separates αC307 from αC324−325 through cleavage sites at the N-terminal positions of αD314 and αD321. This allows better resolution of the modification sites between αC307 and αC324−325, which is not possible in the tryptic fragments. Amino acid sequence information and the mass of each peak are provided in Table 2. The number of asterisks (*) next to the residue number designates the number of NEM-modifications in that peptide.

found in the *β* subunit (Figure 10). Inspection of the mass spectrum of tryptic peptides generated by complete digestion of the same protein revealed localized NEM modifications with 25% of the αC106 peptide modified and a distribution between 1 and 3 NEM-modifications of the αC307, C324−325 peptide.

DISCUSSION

Limited Chymotryptic Digest to Probe Conformational Dynamics. Proteases with high specificity, such as chymotrypsin, yield a relatively well-defined set of peptide fragments upon complete digestion. However, the ensemble of peptide fragments generated by limited proteolysis of a native protein depends on the accessibility of specific amino acid side chains to the protease. That the degree of protease accessibility to these specific amide bonds reflects the dynamic nature of the polypeptide surrounding each labile site under the conditions of the experiment is the premise underlying the use of limited proteolysis as a probe of protein dynamics (*48*). The limited chymotryptic digestion of a partially labeled α*β* heterodimer sequentially generated complementary peptide fragments that were identical to those generated by digestion of the unlabeled heterodimer, showing that *N*,*n*-octylmaleimide (NOM) modification of a limited number of cysteinyl residues does not significantly perturb the conformation of the heterodimer. Since modification with the larger, more hydrophobic NOM did not result in exposure of additional peptide bonds, modification with *N*-ethylmaleimide was presumed to have minimal impact on the structure as probed by limited proteolysis.

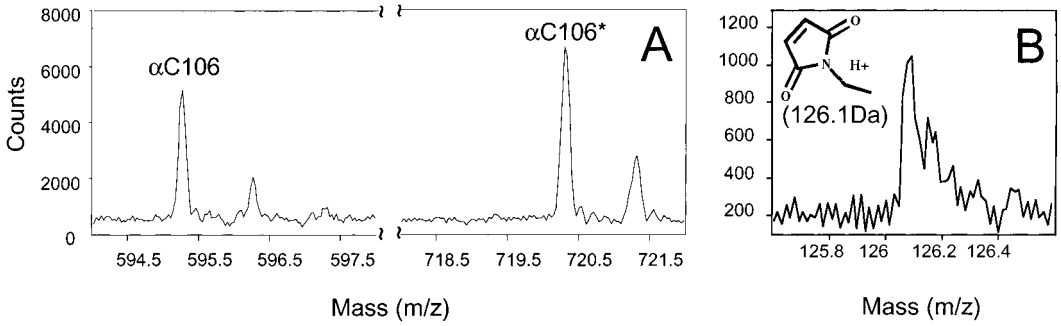

FIGURE 6: Regions of the mass spectrum showing molecular ion peaks of an equimolar mixtures of unmodified and NEM-modified tryptic peptides containing αC106 (α-FGI[106]CR) having different peak area integration values (A). The peak area of the modified form is larger than that of the unmodified form. We hypothesize that *N*-ethylmaleimide modification may have added a protonation site on the modified peptide. A sample of NEM was analyzed and a positively charged ion species was detected at a mass-to-charge (*m*/*z*) ratio of 126.1 Da, corresponding to a protonated NEM (B).

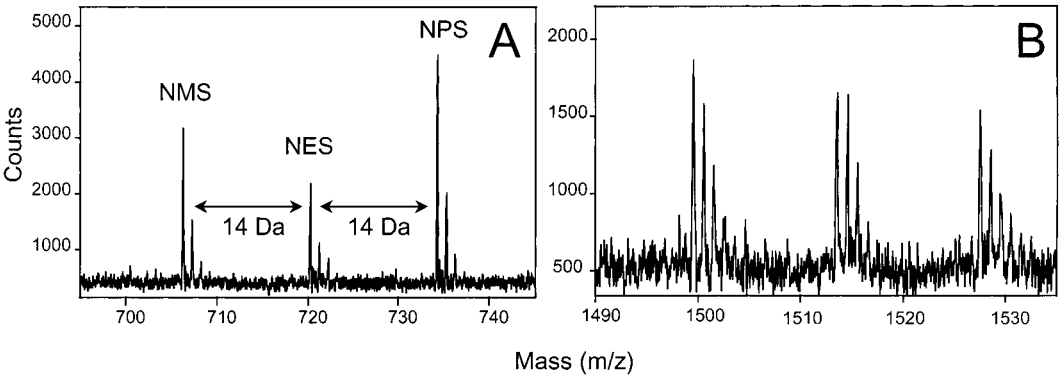

FIGURE 7: Regions of the mass spectrum showing molecular ion peaks of equimolar mixtures of *N*-methylmaleimide, *N*-ethylmaleimide, and *N*,*n*-propylmaleimide modified cysteine-containing tryptic peptides reveal no distinguishable pattern of relative ion abundance. The relative ion abundance of a particular peptide modified by all three alkylmaleimides seems to be highly dependent upon the amino acid sequence of that particular peptide. This is evident in the mass spectrum (A) of the peptide that contains αC106 (αFGI[106]CR) and the spectrum (B) of the peptide that contains αC130 (αALMD[130]CWYDLMK).

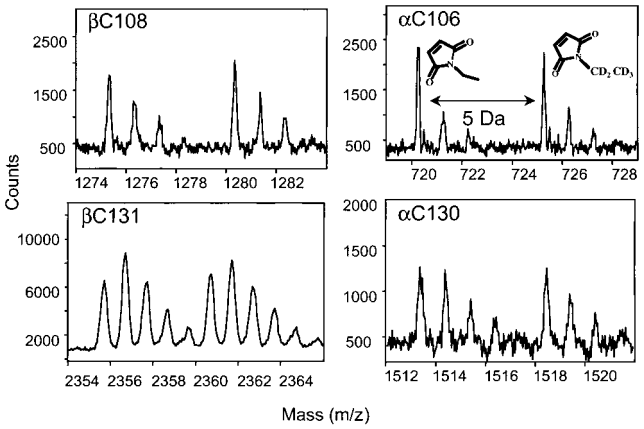

FIGURE 8: Selected regions of the same mass spectrum showing molecular ion peaks with similar peak area integration due to equimolar mixtures of H_5-NEM and d_5-NEM modified tryptic peptides. The mass difference between the two isotopic forms of NEM is 5 Da. This figure demonstrates that there is a linear correlation between the ratio of the peak area integration values and the ratio of the mixture of d_5- and H_5-NEM modified tryptic peptides, allowing the direct quantitation of the extent of modification of each cysteinyl residue in the protein.

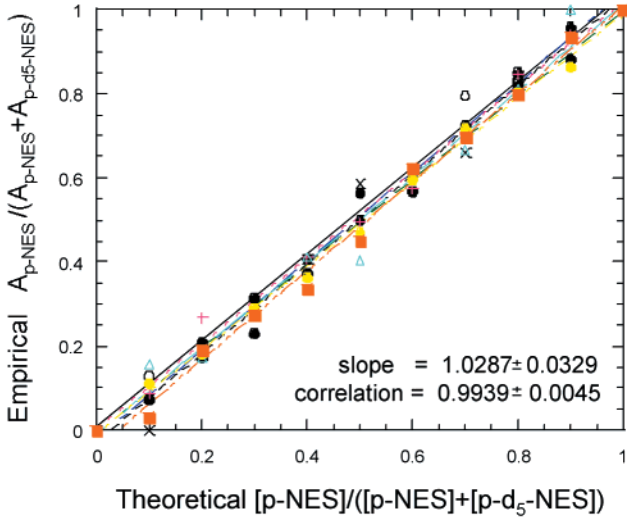

FIGURE 9: Plot of the ratio of the calculated peak area versus the determined ratio of the mixtures of d_5- and H_5-NEM modified tryptic peptides. The good correlation between the concentration ratio $[H_5]/([H_5]+[d_5])$ and the peak area integration ratio $A_{H_5}/(A_{H_5}+A_{d_5})$ for each single cysteine-containing tryptic peptide allows direct quantitative measurement of the extent of modification of each cysteinyl residue in the protein by mass spectrometry.

Qualitative Requirements of PA/MS. Peak identification of the unmodified and *N*-alkylmaleimide-modified forms of the intact subunits and derived proteolytic fragments was required to determine the fractional subpopulation of label incorporation in the protein. The mass spectra of the intact α and β subunits show the extent of incorporated label in the major subpopulation of each subunit, while the complete tryptic digest of these subunits allows the determination of the degree of label incorporation at each cysteine in the protein. Peak identification of all types of cysteine-specific modification of proteolytic peptides is important in the

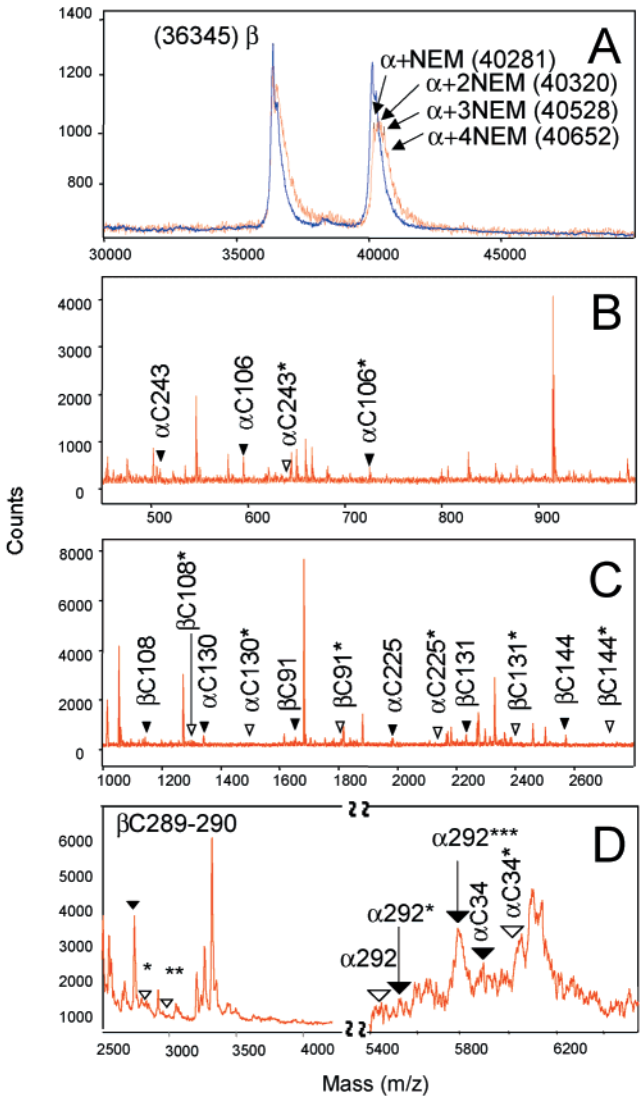

FIGURE 10: Superimposed MALDI-DE-TOF mass spectra of unmodified and partially *N*-ethylmaleimide (NEM) modified forms of *V. harveyi* luciferase subunits incubated in 2 M urea and of the tryptic peptide fragments generated by complete digestion. The mass spectra of the intact subunits show that only the α subunit is partially modified with three NEM (A); the number of modifications was determined by the difference in mass between the unmodified and the modified forms of the subunit divided by the mass of NEM (125 Da). Complete tryptic digestion of the protein was used to identify the specific cysteines that were modified. Amino acid sequence information and the mass of each peak are provided in Table 1. The number of asterisks (*) next to the residue number indicates the number of NEM modifications in each tryptic peptide. The inverted triangles (▼) locate the mass-to-charge (*m*/*z*) positions of the unmodified and the NEM-modified forms of all the cysteine-containing tryptic peptides. A filled triangle indicates the presence of a specific peptide, while a hollow triangle indicates its absence. The modifications were present only at positions αC106 and at αC307, αC324−325. The αC106 site was partially modified (panels B and C), while the αC307 and αC324−325 sites were completely modified (peptides labeled α292 in panel D and Table 1). These results are consistent with the observation that the partially modified intact α subunit (panel A) had two populations, one singly modified and the other with three modifications.

establishment of the relative order of the reactivity of cysteinyl residues in the protein. The order in which cysteines react with the alkylating agent reveals information regarding the environment surrounding each cysteine. Cysteines that are highly susceptible to alkylation are probably located on the surface or in a surface accessible cavity, while those that are less rapidly alkylated are presumed to be buried in the hydrophobic core of the protein. In the case of bacterial luciferase in the presence of 2 M urea, the change in the order of reactivity of the cysteines revealed a dramatic change in the conformation of the protein, significantly increasing the reactivity of some of the cysteinyl residues with minimal impact on others. These observations afford an understanding of the structure of the partially folded heterodimeric protein as it exists in 2 M urea at a level heretofore unavailable.

Quantitative Aspect of PA/MS. A logical extension to the qualitative characteristics of mass spectrometry is the quantitative capability of the technology (*49*−*52*). Accurate measurement of the extent of modification in each cysteine-containing peptide is an important requirement in the determination of the rate of modification derived from equilibrium unfolding/folding experiments. The rate of modification can be determined by monitoring the sequential shift in the mass of cysteine-containing subunits of protein as a function of time, while complete tryptic digestion of the subunits allows a measure of the degree of label incorporation into each cysteinyl residue in the protein. However, it is extremely difficult to obtain reliable quantitative measurements of modification due to poor shot-to-shot reproducibility, lack of homogeneity of the sample spots, and fluctuation in the laser power. In the past decade, several techniques have been developed in conjunction with MALDI-TOF-MS for quantitative analysis of biopolymers. Nelson and McLean (*49*) used horse heart myoglobin (16 951.5 Da) as an internal reference molecular ion peak to normalize the variable molecular ion peaks of different concentrations of horse heart cytochrome *c* (12 360.7 Da). Use of an internal standard permits demonstration of a linear correlation between the absolute concentrations and the normalized molecular ion peak of horse heart cytochrome *c* (*49*). Care should be used in selection of proteins to be used as internal references to make sure that the reference protein is not preferentially ionized so that a weakly ionized analyte protein may not be detected. Preston et al. (*50*) utilized nitrocellulose as a base layer onto which bradykinin in 4-nitroaniline matrix was deposited, resulting in an improvement in the linear correlation between the measured ion yield and the absolute concentration of bradykinin.

We attempted HPLC quantitation of NEM-modified and unmodified peptides of *V. harveyi* luciferase identified by MALDI-TOF-MS, but this approach was exceedingly time-consuming. Measurement of the reactivity of each cysteinyl residue in the protein requires that the ratio of the integrated peak areas between the modified and unmodified cysteine-containing peptides accurately reflect the concentration ratio of peptide mixtures. However, the mass spectrum acquired from equimolar mixtures of modified and unmodified tryptic peptides showed higher ion abundance of the NEM-modified peptides than the unmodified peptides (Figure 6A), suggesting that there is a potential protonation site in *N*-ethyl maleimide causing the increase in ion abundance of the modified peptide. Support for this hypothesis is that the reagent *N*-ethyl maleimide was detected in the mass spectrum at a mass to charge (*m*/*z*) of 126 Da (Figure 6B). By modifying equal amounts of tryptic peptides with *N*-methyl-, *N*-ethyl-, and *N*,*n*-propylmaleimide, we were surprised to find that the chainlength of the alkyl substituent had a nonuniform

effect on the ion yields of each set of peptides (Figure 7). Similar comparisons were carried out with all the thiol-containing peptides in luciferase. There was no obvious correlation with amino acid composition, general chemical properties, or the size of the peptide with the observed effect of the N-alkyl chainlength on ion yield. We resolved the ion-yield problem by using both the deuterated and protonated forms of *N*-ethylmaleimide in a similar mixed-label experiment. We defined the relative ion abundance as the ratio between the peak area integration of a d_5-NEM-shifted peak and the total of the integrated areas of d_5- and H_5-NEM-shifted peaks.

$$\text{relative ion abundance} = A_{d_5\text{-NEM}}/(A_{d_5\text{-NEM}} + A_{H_5\text{-NEM}})$$

This equation is not affected by problems encountered in absolute quantitative MS analysis such as shot-to-shot and sample-to-sample inconsistencies in ion abundance. Since the calculation involves only the relative quantity of isotopically differentiated peptides, it provides its own internal standard and negates irreproducibility in ion signal intensities.

We also defined the concentration ratio as the ratio between the concentration of d_5-NEM-modified peptides and the sum of the concentrations of d_5- and H_5-NEM-modified peptides.

$$\text{concentration ratio} = [d_5\text{-NEM-peptides}]/([d_5\text{-NEM-peptides}] + [H_5\text{-NEM-peptides}])$$

A range of concentration ratios of deutero- and proteo-NEM modified peptides was prepared and analyzed by MALDI-TOF-MS. The resulting correlation and the slope of the line between the relative ion abundance and the concentration ratio over the range of 0.1 to 0.9 were found to be close to 1.0 (Figure 9).

An added advantage of using this combination of labels is that it allows the determination of both the average number of labels incorporated in the intact protein and the extent of modification in each site of the protein in the same sample. By quenching the pulsed-alkylation reaction with a large excess of β-mercaptoethanol, the average number of labels incorporated in the intact subunits of *V. harveyi* luciferase could be determined by the shift in the average mass of the individual subunits; this mass shift should be divisible by the mass of d_5-NEM (130 Da). Addition of trypsin and H_5-NEM to an aliquot of a protein sample that has undergone the cleanup step and pH neutralization with ammonium bicarbonate allows the generation of tryptic peptides with all cysteines modified. The extent of modification is measured by the relative ion abundance of each cysteine-containing peptide. Using this additional feature of the method, we were able to determine the order of the reactivity of the cysteinyl residues in the native conformation of the $\alpha\beta$ heterodimer (αC106 > βC108 > αC130 > βC131 > αC243 > βC91 > αC225 > βC144), providing information about the localized surface accessibility around each thiolate.

PA/MS Cleanup Steps. The removal of high concentrations of salts and urea is required prior to mass spectral analysis. Buffer salts maintain the desired pH of the solution, which affects protein conformation and solubility; however, buffers are also major contaminants that compete with the ionization and detection of peptides and protein in the mass spectral analyses. The urea, used to promote unfolding of protein, also forms a highly ionizable protonated resonance structure with a detectable ion abundance at *m*/*z* of 61 Da. A disposable Amika microtip C4 column provided a time-efficient and easy-to-use method for removing salt buffers and urea. The columns can easily handle large volumes of samples for purification. The high percentage of acetonitrile used for elution also affects both sample deposition and homogeneity of crystallization on the MS target plate, which both affect mass spectral analyses; thus, acetonitrile should be removed from the samples by vacuum evaporation and replaced with water prior to sample deposition.

Probing the Structure of $\alpha\beta$ Heterodimeric Intermediate in 2 M Urea. The studies described above have demonstrated that pulsed-alkylation mass spectrometry (PA/MS) satisfies all the important criteria required for a viable and robust method for probing structural changes in proteins. We tested the methodology to investigate the structural differences between a native enzyme and a folding intermediate that is well populated. The different aspects of the methodology were utilized to probe the urea-induced structural changes in the $\alpha\beta$ heterodimeric intermediate of bacterial luciferase. The results demonstrated that when the protein is in 2 M urea, the cysteinyl residues in the β subunit are not modified. On the contrary, the thiolates in the C-terminal domain of the α subunit (αC307, αC324−325), which are unreactive under native conditions (50 mM phosphate buffer, pH 7), became highly reactive to NEM modification when the protein was incubated in 2 M urea (Figure 10). These new sites of modification were observed in addition to modification of a highly reactive cysteine near the active site of the protein at position α-106 (*53*). The increase in reactivity of the C-terminal cysteinyl residues in 2 M urea provides direct evidence of the unfolding of the C-terminal domain of the α subunit in the heterodimeric intermediate. The above observation is also consistent with the unfolding of the C-terminal domain of free α subunit as reported by Noland et al. (*39*).

The results of the study presented here show that pulsed-alkylation mass spectrometry is a rapid and straightforward method for probing the dynamic structure of intermediates and folded complexes of proteins under a variety of conditions through simultaneous determination of the location and extent of labeling in a protein. Both the qualitative and the quantitative criteria required for this method to be applicable to an array of experimental problems have been satisfied. Reaction conditions and solutes required for the pulsed-alkylation reaction, but detrimental for mass spectral analyses, such as high concentrations of urea, residual alkylating agent, quenching agent, and buffer salts, were removed from the samples by the addition of the sample cleanup step using C4 microtip columns. Combining the information gathered from proteolytic-mass spectral analyses with those from the high-resolution X-ray crystal structure of bacterial luciferase provides new insight into the structural dynamics of the protein.

The basic principle of PA/MS described here is no different than any of a large number of double label methods that have been used for many years to analyze structural differences and similarities between potentially different forms of large biological molecules. The method employed here allows quantitative determination of the rates of reaction

of specific amino acid residues (cysteinyl residues in the present example) with modifying reagents. PA/MS may be widely applicable in probing the reactivity of a set of residues that are important for structure and function of other protein systems. For example, PA/MS has been used to investigate the coordination affinity for zinc within each of the six zinc-finger domains of MRE-binding transcription factor-1 (Zn_6 MTF-zf) in its free and DNA-bound forms yielding structural data heretofore inaccessible. These latter experiments are presented and discussed in the companion paper in this issue [Apuy, J. L., Chen, X., Russell, D. H., Baldwin, T. O., and Giedroc, D. P. (2001) *Biochemistry 40*, 15164−15175].

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