Tuning the Selectivity of Protein Photocleavage: Spectroscopic and Photochemical Studies

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Received December 28, 1998. Revised Manuscript Received March 5, 1999

Abstract: Site-specific photocleavage of lysozyme by *N*-4(1-pyrene)butyroyl-L-phenylalanine (Py-Phe) prompted us to investigate the role of the peptidyl side chain in determining the specificity of the protein photocleavage. The spectroscopic and photocleavage properties of Py-Gly, Py-$(Gly)_n$-Phe ($n = 0, 1, 2$), and Py-Phe-Gly-Gly, in this context, is described here. The linker length/structure of these molecules has a profound effect on the spectroscopic and photocleavage properties of the probes. The absorption spectral changes accompanying the binding of the probes Py-$(Gly)_n$-Phe to the proteins were independent of the linker structure or length. Binding constants of these probes with proteins such as bovine serum albumin (BSA) or lysozyme, varied from $2.2 \pm 0.3 \times 10^5$ dm^3mol^{-1} to $6.5 \pm 0.4 \times 10^7$ dm^3mol^{-1}. Binding constants of Py-Phe and Py-Phe-Gly-Gly for BSA have been an order of magnitude larger than those of Py-Gly, Py-Gly-Phe or Py-Gly-Gly-Phe. The fluorescence spectral changes, in contrast to the absorption changes, depended on the probe structure suggesting the subtle role of the linker structure on the probe binding properties. In addition to the pyrenyl fluorescence, new, broad emission was observed at 466 nm with Py-Phe and Py-Phe-Gly-Gly when bound to BSA, but no such emission was observed with the other probes or with any of these probes bound to lysozyme. Much clearer distinction between the probes can be ascertained from the circular dichroism (CD) spectra. No two CD spectra of the probe−protein complexes were superimposable, clearly demonstrating the differences in the chiral environment surrounding the pyrenyl chromophore in the protein matrix. Fluorescence quenching experiments using Co(III)hexammine (CoHA) as the quencher indicate extensive protection of the fluorophore bound to BSA, while the chromophore bound to lysozyme was relatively more accessible. The tripeptide probes were protected better than the dipeptide probes. The distance of separation between the carboxyl function and the pyrenyl group in these probes has a substantial effect on the accessibility of the probe to CoHA. These differences are, in turn, expected to influence the photocleavage efficiencies. Photocleavage of lysozyme was observed when probe−protein complexes have been irradiated at 344 nm in the presence of CoHA as an electron acceptor. All of the probes showed high specificity with lysozyme and resulted in just two product bands. In case of BSA, the protein cleavage site location and specificity varied drastically with the probe structure, suggesting major changes in the selectivity as a function of probe structure. Peptide sequencing studies of the photofragments from lysozyme revealed the location of the photocleavage sites. While Py-$(Gly)_n$-Phe ($n = 1, 2$) cleave lysozyme at a site between Trp108 and Val109, similar to that for Py-Phe, a second minor cleavage site at Ala110/Trp111 was observed for Py-$(Gly)_n$-Phe ($n = 1, 2$) and Py-PheGlyGly. The ratio of the yields of the major to the minor product, with lysozyme, depended on the nature of the linker. No protein cleavage was observed in the absence of the probe or CoHA or light. The spectral and the photochemical studies indicate the binding and cleavage specificity of the probes vary with the side chain structure.

Protein−ligand interactions are important in key biological processes such as enzyme−substrate recognition, hormone action, signal transmission, and cell communication. Identifying the molecular recognition features involved in these interactions is important for controlling or manipulating these events. Such knowledge will also be useful for the design of reagents that can bind to proteins at specific sites and cleave the protein backbone upon activation with light (photoproteases). Photoproteases can be useful as biochemical tools[1−8] in correlating the protein structure with activity, in the design of new therapeutic agents, and for converting large proteins into smaller fragments that are more amenable for sequencing.

One approach for the design of such photochemical reagents is to link specific recognition elements to photoactivatable chromophores. The choice of the chromophore can be such that its spectral properties are sensitive to the microenvironment, and also, provide a long-lived excited state to initiate specific chemical events. The results from spectroscopic studies of the protein−ligand complexes can be correlated with the protein photocleavage results. The structure of the recognition element can be varied systematically to examine the effect of probe structure on its binding selectivity. Chiral probes are described here[9] that have: (a) high affinities for proteins, (b) intense absorption bands in the 300−400 nm region, (c) long-lived

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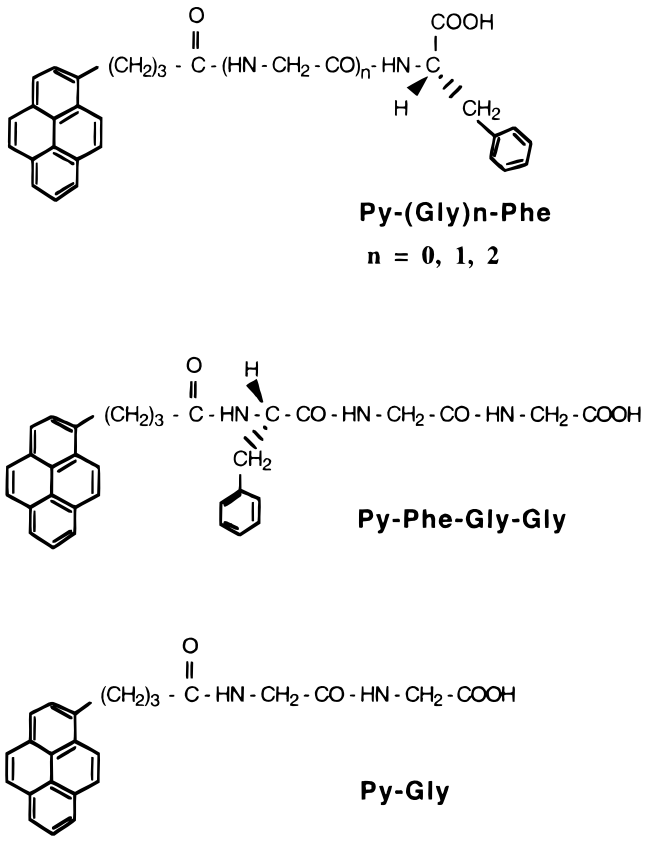
10.1021/ja9844377 CCC: $18.00
Published on Web 04/15/1999

Chart 1. Structures of the Pyrenyl Probes[a]



[a] Distance of separation between the hydrophobic and hydrophilic functionalities is varied by introducing the Gly residues. Py-Phe-Gly-Gly is chosen to test the effect of the location of phenylalanine in the linker on the binding and photocleavage properties.

singlet excited states for the initiation of photoreactions, and (d) a chromophore that has been used to cleave proteins with high selectivity.[10]

Use of light as a reagent for protein cleavage is particularly interesting, and photoreactions have specific advantages over thermal reactions. Photoreactions provide a sharp control for the initiation and termination of the reaction, and visible light is a green reagent. Light-initiated reactions, furthermore, are amenable to flash photolysis studies to investigate mechanistic details. By controlling the wavelength of excitation, specific chromophores can be selectively activated to high energies, thereby minimizing side reactions. Vanadate, for example, has been used as a photoprobe to investigate phosphate binding sites of proteins,[11,12] and our goal has been to develop alternative reagents to vanadate.

Site-specific photocleavage of proteins with a pyrenyl probe was recently reported from this laboratory.[9,10] Covalent linking of 4(1-pyrenyl)butyric acid to the N terminus of L-phenylalanine with *N,N′*-dicyclohexylcarbodiimide led to Py-Phe (Chart 1), containing an asymmetric center. Lysozyme was cleaved exclusively between the residues Trp108 and Val109 by Py-Phe. These residues are at the surface of lysozyme, well-exposed to the solvent, near the known substrate binding site.[10] Photocleavage of lysozyme did not alter its catalytic efficiency, and this observation clearly indicates the negligible effect of the photocleavage chemistry on the protein activity. The cleaved fragments may be reassociating to produce the active enzyme, or the major fragment carrying the active site is nearly as active as the native protein. In either case, the photoreaction was found to be benign. In a similar manner, Py-Phe cleaves BSA between residues Leu346 and Arg347, and these residues are located in a hydrophobic pocket buried in domain II, subdomain B.[13] Py-Phe is the first organic photochemical reagent that exhibits a high selectivity and efficiency for protein cleavage.

Photochemical reactivity of the pyrenyl chromophore and its potential to induce protein strand scission is demonstrated in the above reports. The sequences where the photocleavage occurs in lysozyme and BSA are different, indicating that the cleavage is not dictated by the type of residues at the cleavage site. Py-Phe activity is, thus, in contrast to that of reagents such as cyanogen bromide which is known to cleave at methionine residues. Salt-bridge formation between the carboxy terminus of Py-Phe and arginine residues of the protein at the cleavage site is suspected to be important in the cleavage site recognition by Py-Phe. Accordingly, the C-methyl ester of Py-Phe binds poorly to BSA or lysozyme, and no protein cleavage was observed with either of the two proteins. Perhaps the carboxyl function of the probe anchors the molecule while the pyrenyl chromophore induces the photocleavage at the binding site.

The above observations prompted us to examine whether the protein photocleavage can be induced at different residues by varying the linker length. The distance of separation between the hydrophobic pyrenyl group and the carboxyl terminus in the probes was increased by introducing glycine (Gly) residues (Chart 1). Gly residues are chosen as spacers due to their small size, they are photochemically inert, and the pyrenyl peptides can be readily synthesized by solid-phase synthesis methods. The pyrenyl group is introduced as the terminating residue in the peptide synthesis, allowing us to prepare large quantities of the pyrene-labeled peptides. The role of the phenylalanine side chain in the cleavage chemistry can be inferred by comparing the binding properties of Py-Gly with those of Py-Phe. Similarly, properties of Py-Phe-Gly-Gly will be compared with those of Py-Gly-Gly-Phe to investigate the location of the phenylalanine side chain on the probe activity. The protein binding and photocleavage properties of Py-Phe analogues indicate significant differences. Correlating the structural modifications of the probes with the differences in their spectral and cleavage properties will be useful in learning how the specific recognition features of the probes alter their selectivity and binding behavior.

Lysozyme and BSA are chosen for these studies to compare the reported Py-Phe cleavage sites on these proteins with those of the Py-$(Gly)_n$-Phe derivatives. The X-ray crystal structure of lysozyme is known,[14] and this information is useful to interpret the photocleavage results and the spectral data. Lysozyme is a small hydrolytic enzyme (molecular weight of 14 300), and enzymatic activity of lysozyme can be followed as a function of photocleavage progress to correlate changes in activity with the progress of the photocleavage. BSA is a plasma protein, and hydrophobic ligands such as fatty acids, steroids,

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bilirubin, etc. bind to BSA.[15] Photocleavage results and spectroscopic details of the binding of Py-Phe analogues are reported here.

Materials and Methods

Lysozyme (MW = 14 300), BSA (MW = 66 267) were purchased from Sigma Chemical Co. Protein solutions were prepared by dissolving the appropriate amount of the protein in 50 mM Tris-HCl buffer, pH 7.0. The absorption spectra were recorded on a Hewlett-Packard Model 8453 diode-array spectrophotometer. All probe solutions were prepared fresh and were used within a day. Calibration graphs were constructed using Beer's law, and probe concentrations were restricted to the linear region of the graph. The molar extinction coefficients of the pyrenyl probes were estimated to be similar to that of Py-Phe, 33 000 M^{-1} cm^{-1} at 343 nm. The fluorescence spectra were recorded on a Perkin-Elmer LS5 fluorescence spectrometer interfaced with an Apple Macintosh computer. No excimer emission from Py-Phe and its derivatives was observed in the concentration range 1−50 μM. The absorption and fluorescence titrations were performed by keeping the concentration of the probe constant while varying the protein concentration. For fluorescence measurements, the pyrenyl probe was excited at 345 nm (isosbestic point), and the fluorescence intensity was monitored at 377 nm. The circular dichroism spectra were recorded on a Jasco-710 spectropolarimeter interfaced with a NECA 486 computer. The spectrometer bandwidth was set to 1 nm, and the response time was 4 s. A quartz microcell with a 10-mm path length was used to load the samples. Each spectrum was obtained by accumulating at least three scans. The spectra were analyzed using J-700 system software supplied by Jasco.

Synthesis of the Peptide Probes. Py-Gly was synthesized by coupling 4-(1-pyrenyl)butyric acid (0.5 g) with glycine ethyl ester (0.4 g) using *N*,*N*′-dicyclohexylcarbodiimide (DCC) (0.4 g) as described earlier.[9] The resulting product was hydrolyzed and has been purified by column chromatography (silica gel) using a mixture of chloroform and methanol (5:1). ^{1}H NMR (270 MHz, d_6-DMSO) 7.9−8.2 ppm (m, 9H), 4.5 ppm (exchangeable NH, 1H), 3.8 ppm (d, 2H), 2.2−2.5 ppm (m, 6 aliphatic H); IR (KBr) 3300 cm^{-1} (OH), 1703 cm^{-1} (C═O, acid), 1640 cm^{-1} (C═O, amide); absorption maxima: 313, 326, and 343 nm; fluorescence maxima: 377, 396, and 417 nm (345 nm excitation). Py-Phe was synthesized in a similar manner.[10]

Py-$(Gly)_n$-Phe (n = 1,2) and Py-Phe-Gly-Gly were synthesized using solid-phase synthetic methods, using the N-terminal extension procedure.[16] The first C-terminal residue (N-blocked) was anchored via the carboxyl function to the polymeric support of the column in the automatic synthesizer. N-terminal extension was carried out with the desired sequence of amino acid residues, and 4-(1-pyrenyl)butyric acid was used as the terminal residue. The peptide was cleaved off the column, and products were purified by HPLC (C-18 column) using the solvent gradient from 100% water to 100% acetonitrile (both containing 0.1% trifluoroacetic acid). The solvent was removed under vacuum, and the residue was recovered as pure product. The individual probes were characterized from UV−vis, fluorescence, ^{1}HNMR (270 MHz, d_6-DMSO), and mass spectral data (FAB).

Photochemical Protein Cleavage. Photocleavage reactions were carried out at room temperature in 50 mM Tris-HCl buffer, pH 7.0. The protein solution (15 μM), containing Py-$(Gly)_n$-Phe (15 μM) and $Co(NH_3)_6Cl_3$ (CoHA, 1 mM) (total volume 100 μL), was irradiated at 344 nm (into the pyrenyl absorption band) using a 150-W xenon lamp source attached to a PTI model A1010 monochromator. UV cutoff filter (WG-345; 78% T at 344 nm) was used to remove stray UV light. Irradiated samples were evaporated under vacuum until completely dry for gel electrophoresis experiments.

SDS-Polyacrylamide Gel Electrophoresis and Sequencing. SDS-PAGE experiments were performed by following the literature methods.[17] Loading buffer (24 μL) (containing SDS (7% w/v), glycerol (4% w/v), Tris-HCl (50 mM), mercaptoethanol (2% v/v), bromophenol blue (0.01% w/v, adjusted to pH 6.8 with HCl) was added to the dried protein samples. Protein solutions (8 μL) were aliquoted and heated for 3 min before loading onto the gel. For lysozyme 12% polyacrylamide gels were used, whereas lower concentrations of polyacrylamide gels (8%) were used for BSA. The gels were run by applying 60 V until bromophenol blue passed through the stacking gel. The voltage was then increased to 110 V and the gels have been run for 2.5 h for lysozyme (1.5 h for BSA). For sequencing studies, Novex mini-gels were used to isolate peptide fragments, and after the electrophoresis, the samples were blotted on to Gore-Tex similar to Western blot, as reported.[17b] The sequencing studies were done at Glaxo-Wellcome, Inc. as described for Py-Phe.[10] For product studies, the protein gels were stained with Coomassie blue for 4 h and destained in acetic acid solution (10%) for 4 h. The protein gels were scanned with a Hewlett-Packard scanner (HP ScanJet 4C), and the images were processed with Adobe Photoshop/NIH-Image software. Each band in each lane was digitized, and the intensities of the bands have been integrated. Molecular weight markers were used in each gel for calibration, and the molecular weights of the fragments have been estimated from their mobilities.

Results

Site-specific photocleavage of proteins by Py-Phe was previously reported, and the spectroscopic details along with the protein cleavage properties of Py-Phe analogues are presented here. The distance of separation between the carboxyl function of the probe and the pyrenyl chromophore is varied systematically, in the current studies, to evaluate how these structural features influence the binding properties and photocleavage results of pyrenyl peptides. Current studies build on the early observations of how length of the linker separating the hydrophobic and hydrophilic regions of the probe plays an important role in the protein binding behavior.[18] Spectral and protein photocleavage studies of Py-$(Gly)_n$-Phe (n = 0, 1, 2), Py-Gly, and Py-Phe-Gly-Gly are reported here.

Absorption Titrations. The electronic absorption spectra of the pyrenyl probes are sensitive to the microenvironment surrounding the chromophore. Addition of lysozyme (0 to 10 μM) to Py-Phe (2.5 μM), for example, causes significant hypochromism (26% at 344 nm, Figure 1A) in the 300−400 nm region. The observed absorbance changes thus provide a spectroscopic handle to monitor the binding of the probes to the protein. The peak positions are unchanged upon binding to lysozyme and suggest a fairly polar medium surrounding the pyrenyl chromophore. For example, the absorption maxima of Py-Phe in the presence of lysozyme is similar to that of the probe in methanol or buffer (data summarized in Table 1).

The absorption spectra of Py-$(Gly)_n$-Phe analogues in the presence of BSA show large red-shifts as well as hypochromism, in contrast to spectral results observed with lysozyme. The absorption spectra of Py-Gly-Phe (3 μM), for example, with increasing concentrations of BSA (0−3 μM) are shown in Figure 1B. The absorption spectra indicate several wavelengths where Py-Gly-Phe absorbance is independent of BSA concentration (isosbestic points, at 315, 328, and 345 nm). Such isosbestic behavior strongly indicates the presence of only two types of chromophores absorbing in this spectral region. These chromophores are assigned to the free and protein-bound forms. The observed red-shift and hypochromicity are consistent with a hydrophobic environment surrounding the pyrenyl chromophore.[18] The absorption spectral changes observed with Py-Gly, Py-Phe-Gly-Gly, and Py-Gly-Gly-Phe (see Supporting Information) in the presence of BSA are similar to those

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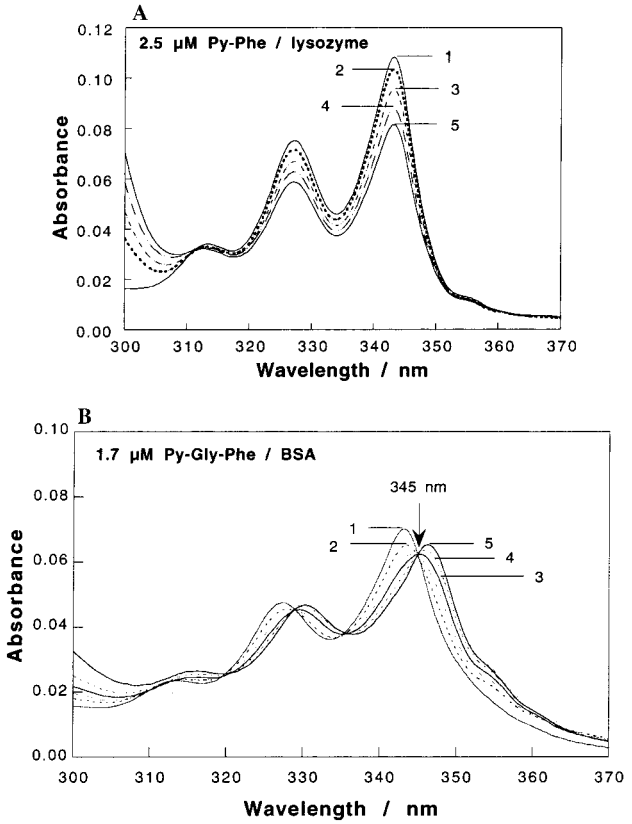

Figure 1. (A) Absorption spectra of Py-Phe (2.5 μM, 1-cm path length) with increasing concentrations of lysozyme: (1) 0 μM, (2) 4 μM, (3) 6 μM, (4) 8 μM, and (5) 10 μM. The absorbance decreases with increasing lysozyme concentration. (B) Absorption spectra of Py-Gly-Phe (1.7 μM) with increasing concentrations of BSA (0 to 4 μM).

observed with Py-Gly-Phe. Similar red-shifts and isosbestic behavior were also observed when Py-Phe binds to BSA.[9] The absorption data thus indicate subtle differences in the probe binding sites on lysozyme and BSA.

Binding Isotherms. With the use of the above spectral data, the binding isotherms for various probes were constructed (Figure 2), following Scatchard analysis (eq 1):[19,20]

$$r/C_f = K_b(n - r) \quad (1)$$

In eq 1, r is the number of moles of probe bound per mole of protein, C_f is the free probe concentration, n is number of binding sites per protein, and K_b is the binding constant. The ratio r/C_f was plotted as a function of r (Figure 2A,B). The binding data were analyzed by using absorbance changes at five separate wavelengths, and the corresponding binding plots resulted in binding constants that varied by 5−8%. The binding constants (K_b) estimated for Py-(Gly)$_n$-Phe with BSA have been in the range of $1.5 \pm 0.3 \times 10^6$ dm^3 mol^{-2} to $6.5 \pm 0.4 \times 10^7$ dm^3 mol^{-2} (Table 2). One binding site per protein molecule ($n = 1 \pm 0.1$, $K_b = 2.2 \pm 0.3 \times 10^5$ dm^3 mol^{-1}) was satisfactory to fit the lysozyme binding data to the Scatchard equation, whereas two binding sites are indicated ($n = 2.2 \pm 0.1$) for BSA. The binding model assumes two independent binding sites per protein, and the resulting fit to the data is shown in Figure 2B. No cooperativity for the binding is apparent. 4(1-Pyrene)-butyric acid, the nonvariant part of these probes, binds only poorly to these proteins, indicating the contributions of the amino acid residues to the overall binding. The absorption spectral changes provide a clear evidence for the binding of the probes to the proteins. The probe fluorescence is also sensitive to the binding of these probes to the target proteins.

Fluorescence Spectra. Addition of lysozyme (0 to 10 μM) to Py-Phe (2 μM) results in weak quenching of pyrenyl fluorescence with no concomitant shifts in the spectral maxima (345 nm excitation, Figure 3A). The spectra were corrected for the decrease in the absorbance accompanying the binding of Py-Phe to lysozyme. Similar spectral results have been observed with Py-(Gly)$_n$-Phe analogues. No excimer emission is noted with lysozyme consistent with a single binding site per protein. Excimers are excited-state dimers produced by the interaction of one chromophore in the ground state with another chromophore in the excited state. Excimer emission, therefore, would have indicated the proximity of two or more chromophores at the probe binding site. The observed 1:1 stoichiometry is consistent with single binding site/single occupancy scenario.

Py-Phe (2 μM) fluorescence, in contrast, is dramatically quenched by BSA (0, 0.5, 1, 1.5, 2, 3, and 4 μM, Figure 3B, excitation at the isosbestic point, 345 nm). The peak positions were unchanged even at the highest protein concentration. Plot of emission intensity in the absence of protein (I_o) to the intensity in the presence of the protein (I) showed a downward curvature (inset in Figure 3B). The plateau occurs when the protein concentration is nearly twice that of the probe concentration. However, a weak, new band at 460 nm is noticeable in the emission spectra with increasing protein concentrations. This new band (Figure 3C) may be due to pyrene excimer emission (excimer $\lambda_{max} = 470$ nm),[21] or the emission could be due to an exciplex (excited-state complex) formed between the pyrenyl chromophore and an aromatic amino acid side chain, such as Trp, present at the binding site. Plot of the ratio of the emission intensity at 460 nm, I_{ex}, to the monomer intensity (377 nm), I_{mono}, showed a plateau at a stoichiometry of 2:1 of BSA to Py-Phe (Figure 3C, inset). Similar broad emission (460 nm) was also observed with Py-Gly-Gly-Phe but not from Py-Phe-Gly-Gly (Figure 3D) or Py-Gly. The 460-nm emission band was more intense with Py-Gly-Gly-Phe than with Py-Phe, under similar conditions. The fluorescence spectral studies thus indicate the subtle differences in the probe binding sites on BSA.

Circular Dichroism Spectra. The absorption and fluorescence spectra of various Py-(Gly)$_n$-Phe analogues are nearly the same, whereas their CD spectra varied dramatically with the probe structure (Figure 4A−C). The CD spectra, therefore, provided a clear method to distinguish between the probes. Binding of Py-Phe and its analogues to proteins, for example, is expected to change the observed CD signals due to the local asymmetric environment provided by the amino acid residues at the probe binding site. Addition of BSA to Py-Phe resulted in dramatic changes in Py-Phe CD spectra (Figure 4A). The strong negative peaks of Py-Phe (336 and 351 nm), for example, have been replaced by positive CD bands at 330 and 345 nm when BSA (50 μM) was added to Py-Phe (50 μM) (thick line). In contrast, only minor changes were observed when lysozyme (50 μM) was added to Py-Phe (50 μM). Negative CD bands similar to those of the free probe but with varying peak intensities at 336 and 351 nm (dotted line) were observed with lysozyme. No CD bands were observed for lysozyme or BSA alone in this wavelength region (300−400 nm, broken and dashed lines for lysozyme and BSA, respectively, Figure 4A).

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Table 1. Extinction Coefficients (ϵ) and Peak Positions of Py-Phe Bound to Proteins and in Various Solvents (Extinction Coefficients Were Measured at λ_{max})

Py-Phe	free	bound to lysozyme	bound to BSA	in MeOH	in benzene
λ_{max} (nm)	343	343	347	342	346.5
ϵ ($\times 10^4$) (M^{-1} cm^{-1}) at λ_{max}	4.3 ± 0.04	3.2 ± 0.2	4.4 ± 0.06	4.8 ± 0.4	5.7 ± 0.06

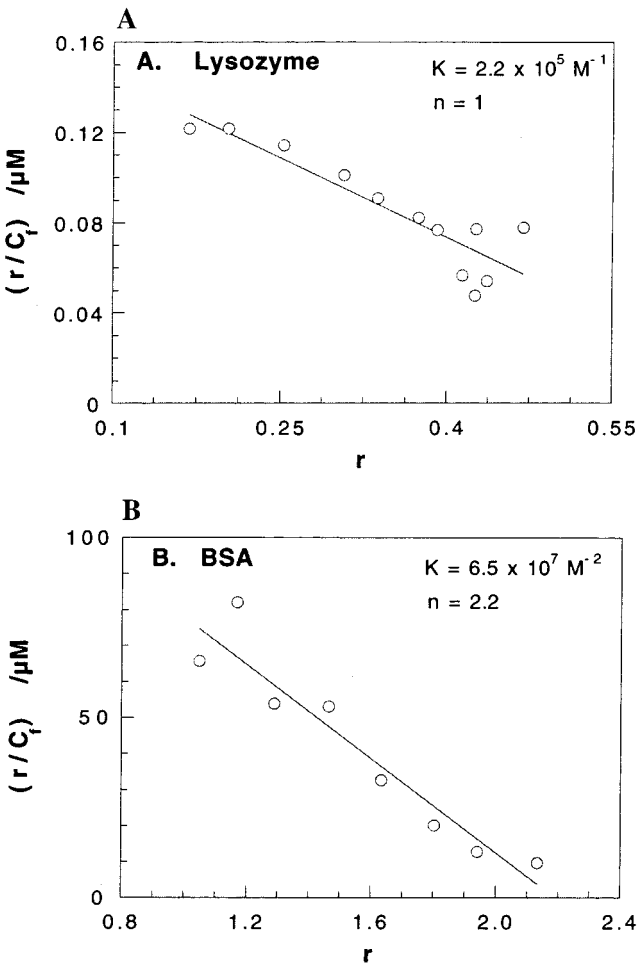


Figure 2. (A) Scatchard plot of Py-Phe (10 μM) at various concentrations of lysozyme (2−20 μM). The data were recorded at five different wavelengths, and the binding constants were averaged. (B) Scatchard plot of Py-Phe (0.5 μM) at various concentrations of BSA (0.1−0.5 μM). The data were recorded at five different wavelengths, and the binding constants were averaged to give 65/μM.

Asymmetry of the microenvironment surrounding the pyrenyl chromophore, therefore, changes upon binding to the proteins, and the CD spectra of Py-Phe bound to BSA is also distinguishable from that of Py-Phe bound to lysozyme.

CD spectra of Py-Gly, Py-Gly-Phe, Py-Gly-Gly-Phe, and Py-Phe-Gly-Gly, similarly, show marked changes when bound to the proteins. Py-Gly has no asymmetric centers and hence shows no CD in the absence of the protein while strong induced CD bands were observed in the presence of BSA (Figure 4B, thick line). No such induced CD was observed with lysozyme. The CD spectral changes observed with Py-Phe-Gly-Gly in the presence of BSA are in contrast to those of Py-Phe (Figure 4C), indicating the differences in the binding sites. These results clearly demonstrate the high sensitivity of the CD spectra to the chiral microenvironment surrounding the pyrenyl chromophore. No two probes shared the same spectra, and both proteins indicate distinct chiral environments for the probes.

Absorption, emission, and CD spectral studies, thus, clearly indicate intimate association of the pyrenyl chromophore with the proteins and that the probes sense different chiral environments, depending on the structure of the probe. Two extreme possibilities for the binding of these probes to proteins can be considered. Binding may occur on the protein surface such that the pyrenyl chromophore is exposed to the solvent. Or that the pyrenyl chromophore is buried in the protein matrix shielded from the aqueous phase (Chart 2). These possibilities are investigated in fluorescence quenching studies.

Fluorescence Quenching Studies. Accessibility of the protein-bound pyrenyl chromophore to $Co(NH_3)_6^{3+}$ (CoHA) was investigated in fluorescence quenching experiments.[22] These quenching studies are relevant because the protein photocleavage is initiated when Py excited state (Py*) is quenched by Co(III) complexes. The ratio of intensity of fluorescence in the absence of the quencher (I_o) to the intensity in the presence of the quencher (I), was plotted according to eq 2,[23] where, K_{sv} is the Stern−Volmer quenching constant.

$$I_o/I = 1 + K_{sv}[Q] = 1 + k_q\tau[Q] \qquad (2)$$

The Stern−Volmer quenching plots for Py-Phe with various concentrations of CoHA are shown in Figure 5. Py-Phe (2 μM) fluorescence is rapidly quenched by CoHA, in the absence of the protein, with a K_{sv} value of 1.9×10^3 dm^3 mol^{-1} (open squares). In the presence of lysozyme (2 μM) the quenching slope was reduced to 1.4×10^3 dm^3 mol^{-1} (open circles), and in the presence of BSA (2 μM), Py-Phe fluorescence is extensively protected by the protein matrix ($K_{sv} = 0.2 \times 10^3$ dm^3 mol^{-1}, closed triangles). Only minor changes in fluorescence lifetimes of Py-Phe were observed when the protein solution was added to Py-Phe ($\tau_{free} = 107$ ns, $\tau_{bound} = 101$ ns, BSA). The changes in the observed K_{sv} values, therefore, reflect the differences in the degree of access of Py-Phe to CoHA. While the lysozyme-bound probe is accessible to CoHA, the BSA-bound probe is well-protected.

Fluorescence quenching data, similarly, with Py-Phe analogues obtained with CoHA have been collected in Table 3. The K_{sv} values for the probes bound to lysozyme are strikingly greater than those of the corresponding BSA-bound probes. Lysozyme-bound probes, therefore, are consistently more accessible to the quencher, whereas the BSA-bound probes are buried in the protein matrix. Probes bound to BSA, however, show an interesting trend. Quenching constants decreased with increased linker length. Both tripeptides showed markedly lower quenching constants than the dipeptide probe. The quenching data are clearly consistent with the hydrophobic burial of the Py group, and the extent of burial seemingly depends on the linker length. The protein bound pyrenyl probes are light-activated to cleave the protein backbone to test how the intervening Gly residues of the probes alter the cleavage specificity of the probes.

Protein Photocleavage Studies. Facile photocleavage of lysozyme and BSA was observed with these pyrenyl peptide probes. Lysozyme, for example, was cleaved by all of the above probes and resulted in two clean fragments of molecular weights

(22) Kumar, C. V.; Tan, W. B.; Betts, P. *J. Inorg. Biochem.* **1997**, 28, 177; Gafney, H. D.; Adamson, A. W. *J. Phys. Chem.* **1972**, *76*, 1105.
(23) Stern, O.; Volmer, M. *Phys. Z.* **1919**, *20*, 183.

Table 2. Binding Parameters for Py-Gly-Phe Analogs with Bovine Serum Albumin

probe	Py-Phe	Py-Phe-Gly-Gly	Py-Gly-Phe	Py-Gly-Gly-Phe	Py-Gly
K_b/M^{-2} (with BSA)	6.5×10^7	1.5×10^7	1.5×10^6	1.5×10^6	1.7×10^6

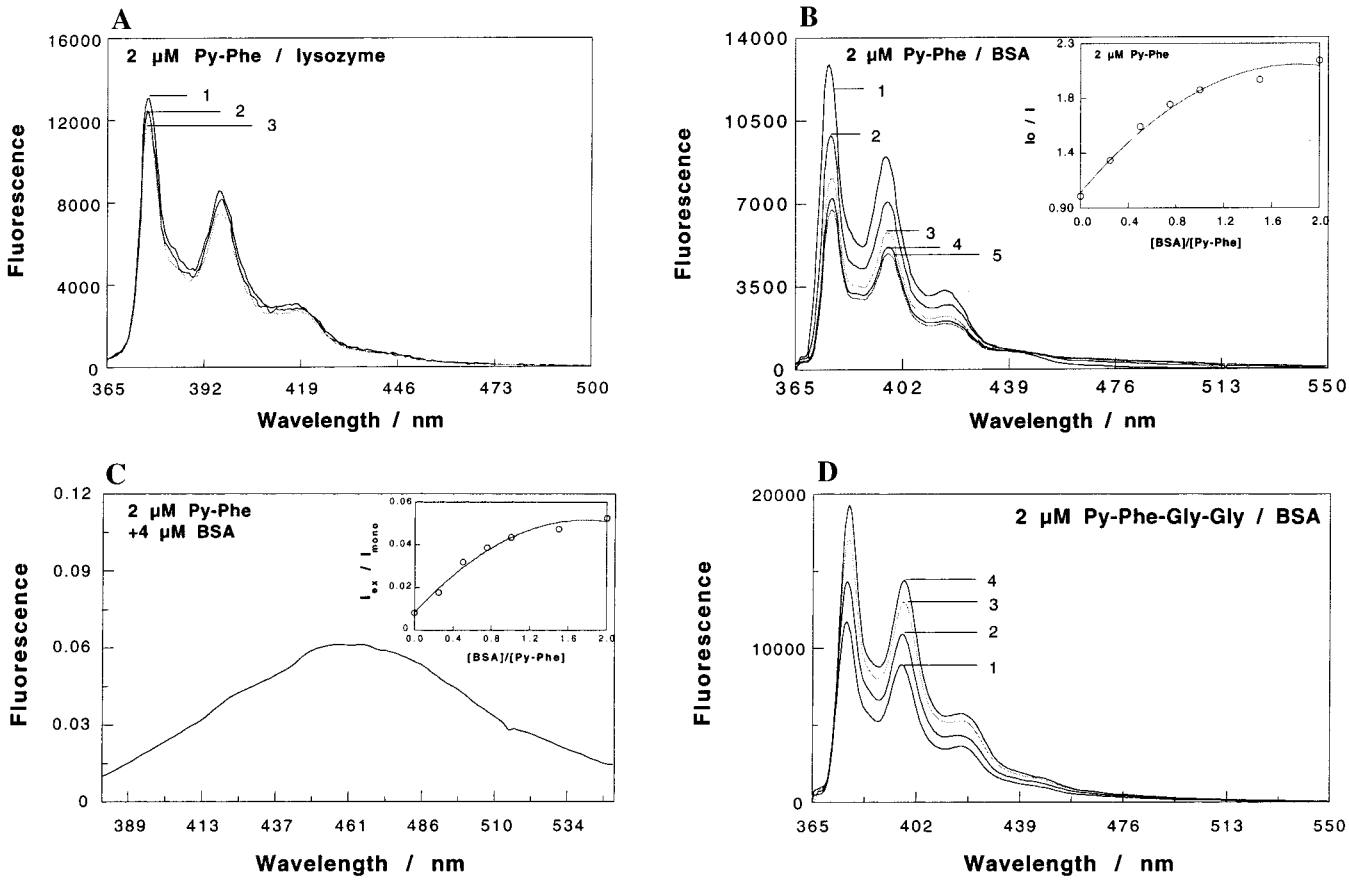


Figure 3. (A) Fluorescence spectra of Py-Phe (2 μM) with increasing concentrations of lysozyme (0−10 μM: (1) 0 μM, (2) 4 μM, and (3) 10 μM). (B) Fluorescence spectra of Py-Phe (2 μM) recorded at various concentrations of BSA (0−2 μM: (1) 0 μM, (2) 0.5 μM, (3) 1 μM, (4) 1.5 μM, and (5) 2 μM). The excitation wavelength was at the isosbestic point, 345 nm. Plot of I_o/I vs [BSA]/Py-Phe ratio is shown at the top right corner. (C) New, broad emission of Py-Phe (2 μM) in the presence of BSA (4 μM), centered at ~460 nm. The plot of I_{ex}/I_{mono} is shown as the inset of the figure. The intensity of the 460-nm band is more prominent with Py-Gly-Gly-Phe than with Py-Phe. (D) Fluorescence spectra of Py-Phe-Gly-Gly (2 μM) with increasing concentrations of BSA, after subtraction of the monomer emission (0−4 μM: (1) 0 μM, (2) 0.4 μM, (3) 3.2 μM, (4) 4.0 μM BSA).

11 and 3 kDa (Figure 6A). Only two product bands were observed irrespective of the linker length or structure. All of the probes resulted in product bands that have the same mobilities. The cleavage site on lysozyme, therefore, appears to be independent of the probe structure. No photocleavage was observed without CoHA, light, or the probe. Most likely, the cleavage occurs at the same general location on lysozyme, if not exactly at the same site.

Photocleavage of BSA by various Py-$(Gly)_n$-Phe analogues, in contrast, indicates a clear dependence of the location of the cleavage site and photocleavage efficiency on the probe structure (Figure 6B). The specificity for photocleavage was dramatically lowered with Py-Phe analogues when compared to that of Py-Phe. Small changes in the probe structure may alter the preferred binding sites on the protein, and subsequent changes in the photochemistry may lead to the drastic reduction in the yield as well as the specificity. The photocleavage yields are lowered due to the decrease in accessibility of the probe to CoHA.

At least three product bands (diffuse) are apparent for Py-Gly (lane 4), while one sharp band just below the unreacted BSA band was noted for Py-Gly-Phe (lane 5). Two bands, weak but similar in mobility to those in lane 3, were observed for Py-Gly-Gly-Phe (lane 6), while two broad bands that smear along the lane were observed for Py-Phe-Gly-Gly (lane 7). Irradiation of BSA in the absence of the probe or CoHA did not yield any fragmentation (lanes 2, 8, 9). Therefore, the pyrenyl probe, CoHA, and light are essential for the protein photocleavage. These experiments establish the ability of Py chromophore to cleave the protein backbone under photochemical conditions, although the cleavage efficiency and location depends on the probe structure.

Amino Acid Sequencing Studies. Due to the ease of separation and purification of the fragments in good yields from lysozyme, the peptide fragments from lysozyme have been subjected to sequencing studies. N- and C-terminal sequencing studies of lysozyme fragments obtained from the photocleavage studies with Py-$(Gly)_n$-Phe, and Py-Phe-Gly-Gly were performed to determine the cleavage site. From the N-terminal sequencing studies, the 11 kDa fragment was identified as the N-terminal peptide of lysozyme with the sequence LysValPheGly. This sequence matches with that of the known N terminus of lysozyme. The N-terminal sequence of the 3 kDa fragment was found to be the internal sequence, ValAla(Trp)Arg. The C-terminal sequencing of the 11 kDa fragment did not yield any

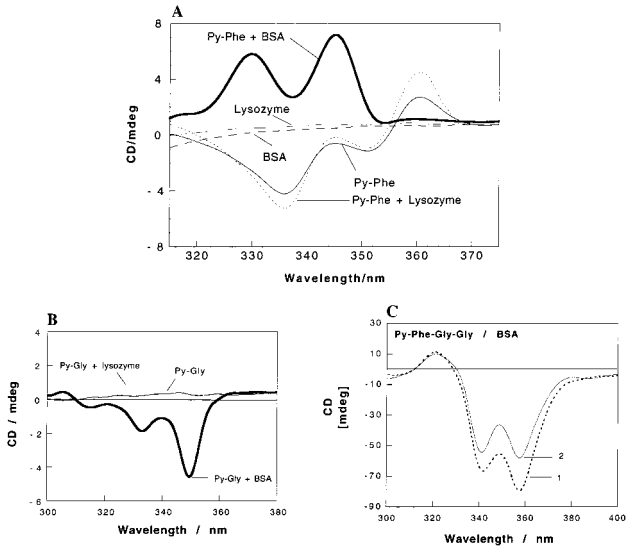
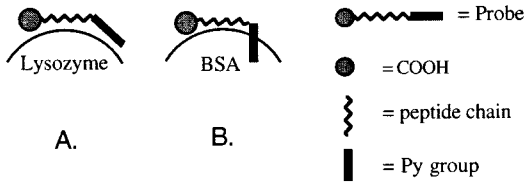


Figure 4. (A) Circular dichroism (CD) spectra of Py-Phe (50 μM) (thin line) shows negative CD bands at 336 and 351 nm. In the presence of lysozyme (50 μM), similar negative CD bands at 336 and 351 nm are observed with increase in the intensity (dotted line). In the presence of BSA (50 μM), positive CD bands at 330 and 345 nm are observed (thick line). Both lysozyme and BSA do not exhibit CD bands in this region (broken and dashed lines, respectively). (B) Circular dichroism spectra of Py-Gly (50 μM) in the absence (thin line) and presence of lysozyme (50 μM, dotted line) or BSA (50 μM, thick line). (C) Circular dichroism spectra (top) of Py-Phe-Gly-Gly (50 μM) in the absence (dotted line (1)) and presence of BSA (2) (50 μM, solid line (2)). No changes in the CD spectra were observed with lysozyme. Red-shifts of the absorption spectra (bottom) of Py-Phe-Gly-Gly (1) (1.4 μM) was observed in the presence of BSA (2) (3.6 μM), with an isosbestic point at 345 nm.

Chart 2. Binding Model Illustrating the Surface and Interior Binding of the Probe with Distinct Accessibilities of the Py Chromophore to the Solvent



interpretable signal. However, the C terminus of the 3 kDa fragment was observed to be Leu, the C-terminal residue of lysozyme. Therefore, the cleavage site was determined from the known primary structure of lysozyme to be between Trp108 and Val109 (Chart 3). An additional minor cleavage site between Ala110 and Trp111 was also observed with Py-(Gly)$_n$-Phe (n = 1, 2) and Py-Phe-Gly-Gly, although Py-Phe cleaves lysozyme at a single site. The ratio of the yield of the major cleavage site to that of the minor is found to be 6:1 for Py-(Gly)$_n$-Phe (n = 1, 2) and 4:1 for Py-Phe-Gly-Gly. Therefore, the photocleavage selectivity and the cleavage site location on lysozyme depends on the probe structure.

Discussion

Chemical proteases are useful in targeting specific sites of proteins,[1] in protein structural studies,[2] and in exploring the exposure of membrane proteins to the aqueous phase.[4] Developing new and highly selective chemical proteases (as biochemical tools) could also contribute to the understanding of how small molecules may interact with proteins.

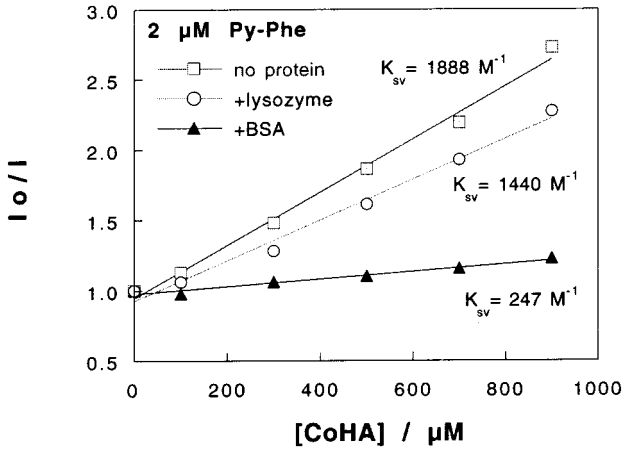


Figure 5. The Stern−Volmer quenching plots from the fluorescence titration data for Py-Phe (2 μM) by CoHA, in the presence and absence of lysozyme or BSA. The fluorescence quenching constants (K_{sv}) were estimated from the plots.

Water soluble globular proteins have hydrophilic functions on the exterior and hydrophobic groups in the interior. Therefore, probes featuring hydrophobic and hydrophilic functionalities are likely to bind selectively at the hydrophobic/hydrophilic interfaces of proteins. Binding will be most favored, in general, at a site where the array of microscopic environments of the binding site are complementary to that of the probe. Probes containing structurally contrasting features (hydrophobic and charged functions) connected through a short tether are more likely to show a higher binding selectivity (Chart 2) when compared to probes carrying a single functionality.[18,24,25] The binding, for example, can be such that the hydrophobic part of the probe is buried away from the aqueous phase, while the charged group is interacting with complementary charged residues at the protein surface. The binding free energy for such a multisite binding model is expected to be more favorable than that for the model for binding to either of the two sites and to improve selectivity. Such multiple site binding appears to be the basis for the observed specificity of Py-Phe. The carboxyl function of Py-Phe is implicated in salt-bridge formation with a neighboring Arg side chain at the binding site.[10] By varying the length of the peptide tether, therefore, the pyrenyl group may be positioned farther and farther away from the anchoring salt bridge. In such a scenario, different residues on the protein may be targeted for cleavage.

Absorption and fluorescence spectral data clearly indicate the binding of the peptide probes to both lysozyme and BSA with binding constants in the range 10^6-10^7 M^{-1}. Binding affinity varies only marginally with probe structure. With BSA, insertion of one or two Gly residues in Py-Phe decreased the binding nearly 40-fold, and the binding constant is the same as that of Py-Gly. Addition of two Gly residues at the end decreased the binding only 4-fold. 4(1-Pyrene)butyric acid, by itself, shows only weak affinity for BSA, further suggesting the subtle roles played by the amino acid residues and their order on the overall binding interaction with BSA. While a single binding site on lysozyme is shared by the peptide probes requiring a 1:1 stoichiometry, the binding site location on BSA appears to vary with probe structure (1:2 stoichiometry of BSA to probe). These observations are consistent with multiple hydrophobic binding sites known for BSA.[18,15] The binding site of BSA appears to be buried at the hydrophobic domains of the protein whereas

(24) Kumar, C. V.; Tolosa, L. M. *FASEB J.* **1993**, *7*, A1131.
(25) Kumar, C. V.; Asuncion, E. H. *J. Am. Chem. Soc.* **1993**, *115*, 8547.

Table 3. Fluorescence Quenching Constants for Py-$(Gly)_n$-Phe Analogs with Lysozyme and Bovine Serum Albumin (CoHA as a quencher) (Extensive Protection with BSA and Marginal Protection Due to Lysozyme Are Evident from the Data)

probe	Py-Phe	Py-Gly	Py-Gly-Phe	Py-Gly-Gly-Phe	Py-Phe-Gly-Gly
K_{sv}/M (free probe)	1.9×10^3	1.3×10^3	1.3×10^3	1.9×10^3	1.3×10^3
K_{sv}/M (with 2 μM BSA)	247	310	376	<10	<10
K_{sv}/M (with 2 μM lysozyme)	1.4×10^3	1.2×10^3	1.0×10^3	650	822

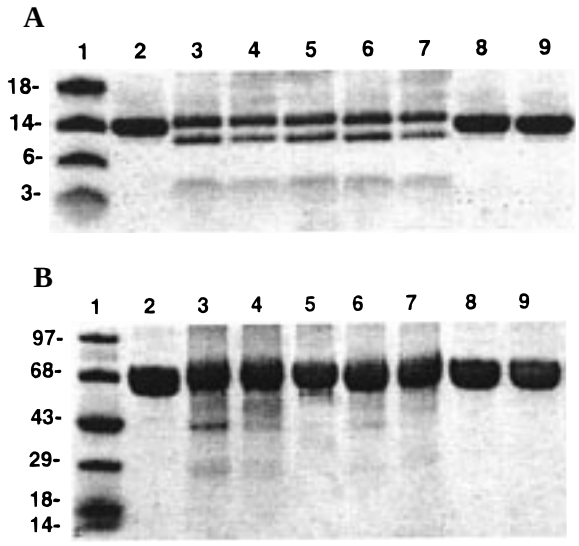


Figure 6. (A) Photocleavage of lysozyme with Py-Gly-Phe analogues. Control lanes contained no light (lane 2), no probe (lane 8, 20 min irradiation), or no CoHA (lane 9, 20 min irradiation), indicating that all three are required for the reaction. Lane 1 contained molecular weight markers as indicated in kDa; lane 3−7, lysozyme (15 μM), CoHA (1 mM), and contained Py-Phe (15 μM) (lane 3), Py-Gly (lane 4), Py-Gly-Phe (lane 5), Py-Gly-Gly-Phe (lane 6), and Py-Phe-Gly-Gly (lane 7), after 20 min of irradiation at 344 nm. The samples were run from top to bottom, and the new bands in lanes 3−7 correspond to the lower molecular weight fragments. Py-Phe (15 μM) was present in lanes 2, 3, and 9. (B) Photocleavage of bovine serum albumin with Py-Gly-Phe analogues. Control lanes contained no probe, no light (lane 2), no probe (lane 8, 60 min irradiation), or no CoHA (lane 9, 60 min irradiation), indicating that all three are required for the reaction. Lane 1 contained molecular weight markers as indicated in kDa; lane 3−7, BSA (15 μM), CoHA (1 mM), and contained Py-Phe (15 μM) (lane 3), Py-Gly (lane 4), Py-Gly-Phe (lane 5), Py-Gly-Gly-Phe (lane 6), or Py-Phe-Gly-Gly (lane 7), after 60 min of irradiation at 344 nm.

the lysozyme binding site is exposed to the solvent. Such a scenario is also consistent with the red-shifted absorption spectra of the BSA-bound probes, while no shifts in the absorption spectra were observed with lysozyme.

Fluorescence quenching experiments indicate that the binding site on lysozyme appears to be at the surface, and the probe is accessible to the quenchers present in the aqueous phase. In contrast, extensive protection of the probe bound to BSA was indicated in the fluorescence quenching experiments. The broad, weak emission observed at 460 nm with Py-Phe and Py-Gly-Gly-Phe (bound to BSA) is perhaps due to excimer emission.[21] Such an explanation is consistent with the observed stoichiometry (1:2, BSA to probe) if both chromophores are binding at the same site. The weak intensity of the 460-nm band and its location suggests another possibility. Exciplex formation between Py and an aromatic residue at the binding site can explain the weak broad emission at 460 nm. While the identity of the residue responsible for the exciplex is not clear, no such emission was observed with Py-Gly, Py-Gly-Phe, or Py-Phe-Gly-Gly bound to BSA, indicating different probes may be binding at different hydrophobic locations on BSA.

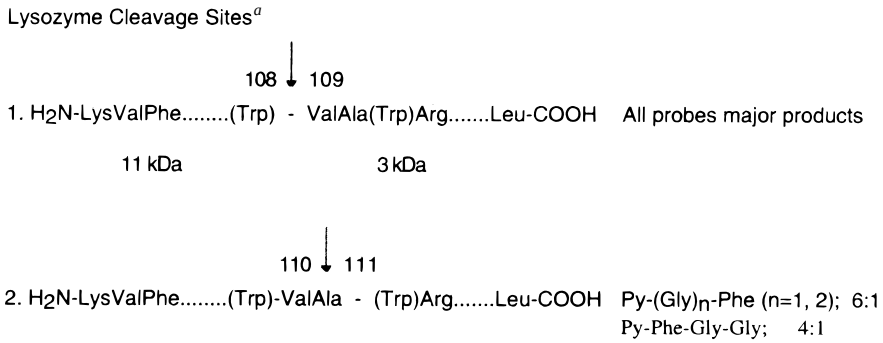
The strong CD bands of Py-Phe analogues due to the phenylalanine asymmetric center are sensitive to the chiral microenvironment (Figure 4). Two distinct contributions to the observed CD changes can be suggested. Orientation of the pyrenyl chromophore with respect to the phenylalanine asymmetric center in the bound probe can be different from that of the free probe due to changes in the side chain conformational equilibrium. This can result in a CD spectrum for the bound probe which is different from that of the free probe. Indeed, no two CD spectra of probe−protein complexes are superimposable. Orientation of the Py chromophore with respect to the phenylalanine asymmetric center is likely to be controlled by the geometry and flexibility of the binding site. Another contribution to the CD spectrum can arise from the asymmetric environment due to the protein side chains. This aspect is clearly evident from the induced CD spectrum of Py-Gly bound to BSA (Figure 4B). This latter contribution will be dictated by specific residues that surround the binding site and their orientations with respect to the pyrenyl chromophore. Indeed, the induced CD bands are indicative of strong interactions between the chromophore and the surrounding protein matrix.[26] These various studies clearly establish the differences in the binding of Py peptides to lysozyme and BSA, their binding stoichiometries, and solvent accessibilities when bound to the proteins.

The binding properties of peptide probes with respect to lysozyme and BSA differ considerably, as pointed out earlier. In case of lysozyme, a single binding site for all of the probes is consistent with the observed spectroscopic and photocleavage results. Essentially, the cleavage occurs at the same site on lysozyme with all of the probes, and an additional site was observed with probes carrying longer tethers. Only two major fragments are produced for lysozyme with molecular weights of 11 and 3 kDa. The major cleavage site was found to be at Val108-Trp109, for all of the probes examined. Increased linker length is expected to systematically move the cleavage site farther away from the anchoring salt bridge. Such a model predicts the photocleavage to occur at different sites for these probes. The second cleavage site was indeed observed with three of the probes. The minor cleavage site (between Ala110 and Trp111) observed with Py-$(Gly)_n$-Phe ($n = 1, 2$) and Py-Phe-Gly-Gly, therefore, supports the differences in the residues that are in contact with the pyrenyl chromophore.

The major cleavage site observed in lysozyme, however, is part of a β-bend, and these residues are not rigidly arranged as in an α-helix or a β-sheet. Perhaps the Val108-Trp109 is a hot spot and is more reactive than the minor site at Ala110-Trp111, while the other residues at this site may be much less reactive. These results may suggest some movement of the reactive species along the peptide chain, and the reactivities of various residues may differ considerably. The location of the second cleavage site down-stream from the major cleavage site suggests that Arg112 may not be the residue involved in salt-bridge formation with the pyrenyl probes. The high yield observed with lysozyme (42−47%) is consistent with the facile access of the

(26) Garnier, F.; Youssoufi, H. K.; Srivastava, P.; Yassar, A. *J. Am. Chem. Soc.* **1994**, *116*, 8813; He, X. M.; Carter, D. C. *Nature* **1992**, *358*, 209.

Chart 3



[a] The first (major) cleavage site of lysozyme was found to be between Trp108 and Val109 using Py-(Gly)$_n$-Phe and Py-Phe-Gly-Gly. The second (minor) cleavage site was found to be between Ala110 and Trp111. The ratio of the first and second cleavage sites is 6:1 for Py-(Gly)$_n$-Phe (n = 1, 2) and 4:1 for Py-Phe-Gly-Gly.

lysozyme-bound probes to the aqueous medium (Table 3) as indicated in the fluorescence quenching experiments.

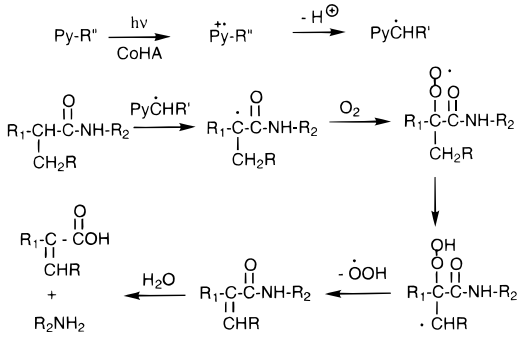
In case of BSA, each probe appears to bind at different locations on the protein and induce photocleavage at the corresponding binding site, changing the cleavage site location and yield. The photocleavage conversions are low with BSA, consistent with the poor access of the probe to the aqueous phase quencher (fluorescence quenching experiments). While a single cut was observed with Py-Phe between Arg346-Leu347 of BSA, the selectivity observed with Py-Phe analogues varied from probe to probe. At least two major cleavage sites can be identified, for the BSA photoreaction, in addition to the known site of Py-Phe on BSA. A new product band was observed for Py-Gly at ~50 kDa. Photocleavage of BSA by Py-Phe analogues is less efficient, and selectivities are lower when compared to that of Py-Phe (Figure 6B). Due to the low conversions and smears observed (Figure 6B), sequencing studies with BSA were not attempted.

The differences in the nature of the binding sites on BSA for these probes is clear from the spectral studies. The data clearly demonstrates the variability in the target site, depending on the probe structure. Thus, a single reactive site present in BSA is not the underlying reason for the high specificity observed with Py-Phe. Perhaps certain residues are more reactive than others, but binding of the probe at or near the reactive site is essential for the photocleavage to proceed.

Light and CoHA are needed for the protein photocleavage, and the photocleavage proceeds most likely via $Py^{+\bullet}$ as proposed.[10] The pyrene cation radical is produced when the probe excited state is oxidatively quenched by the Co(III)HA. Flash photolysis studies, reported earlier, provided direct evidence for the pyrene cation radical formation.[10] The cation radical may undergo deprotonation to result in the corresponding radical. Hydrogen atom abstraction by the radical from the peptide backbone, followed by backbone cleavage can lead to fragmentation (Chart 4). While details of these steps are likely to be more complex than envisioned here, model studies are in progress to examine the proposed protein cleavage mechanism. The mechanism is to be consistent with the viability of the N-terminal sequencing of the newly formed peptide fragments.

Current results also prove that the reactive intermediate produced in the protein matrix does not migrate great distances and cleave at other sites. This is because the diffusional characteristics of the intermediates produced from these probes are expected to be similar, and if diffusion is significant, all of the probes would have shown multiple cleavage sites. Photocleavage results clearly show the subtle role of the intervening Gly residues on the selectivity. Efficiency of the photocleavage also appears to depend on the probe structure. This is most likely due to the differences in the proximity of the pyrenyl moiety to the reactive site, quenching of the excited state by the nearby residues, and interception of the cation radical by residues leading to dead-end products that may not lead to photocleavage. Attachment of known recognition elements to the pyrenyl chromophore will aid us in the rational design of chemical reagents to target specific sites of proteins. Protein scissors that can be activated with light complement the photoaffinity methods and extend our ability to target desired sites on proteins. In addition to biochemical applications, such reagents may be of interest for therapeutic purposes.

Chart 4. Formation of Py Cation Radical and the Subsequent Reaction with the Peptide Backbone Is Shown[a]



[a] Hydrogen abstraction, followed by reaction with oxygen, is one of the possible mechanisms of protein cleavage. The newly produced amino terminus is amenable for sequencing experiments.

Acknowledgment. We are grateful for Mary B. Moyer and William Burkhart, Glaxo Wellcome, Inc. for the amino acid sequencing data. The financial support of University of Connecticut Research Foundation, the Petroleum Research Fund (ACS-PRF#33821-AC4) and the National Science Foundation (DMR-9729178) are gratefully acknowledged.

Supporting Information Available: Absorption spectra of Py-Gly, Py-Gly-Gly-Phe, and Py-Phe-Gly-Gly (2 μM) in the presence of BSA (0-3.6 μM) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA9844377