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Photochemical Protein Scissors: Role of Aromatic Residues on the Binding Affinity and Photocleavage Efficiency of Pyrenyl Peptides

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Abstract—Photocleavage of proteins by a series of organic probe molecules is examined as a function of probe structure. For example, pyrenyl peptides Py-Gly-X (X=Trp, Tyr, Phe, and His, and Py=4(1-pyrenyl)butyroyl)) are prepared, and their protein binding/photocleavage properties have been examined. The binding constants with bovine serum albumin (BSA) are in the range of 10^7 to 10^5 and binding of the probes to the proteins is evident in absorption, fluorescence and circular dichroism experiments. While the fluorescence of Py-Gly-Tyr increases upon binding to BSA, quenching is observed with Py-Gly-Trp or Py-Gly-His. While hyperchromism is observed with Py-Gly-Trp/BSA, hypochromism is the norm for all the other probes with this protein. Binding of all the probes to BSA or lysozyme resulted in major changes in the circular dichroism spectra of the probes. Photoexcitation of the probe/protein complexes, in the presence of an electron acceptor, resulted in protein photocleavage. The phenylalanine and histidine analogs resulted in photocleavage of both BSA and lysozyme while the tyrosine and tryptophan analogs did not yield any fragmentation with either of the two proteins. The photocleavage sites are similar to those reported for Py-Phe. Flash photolysis studies of the probe/protein mixtures indicate that the initially produced pyrene cation radical is strongly quenched by the tyrosine and tryptophan residues and resulted in the corresponding amino acid radicals. Strong modulation of the photoreactivities of the probes by specific residues of the probe provide insight in learning how the photocleavage efficiencies of the probes can be improved in future studies.

Introduction

The development of site-specific protein cleaving reagents has been of recent interest in biological chemistry. Because of the extreme stability of the peptide bond (half life of ~7 years at room temperature, pH 7),[1] the development of peptide bond cleaving reagents (artificial peptidases) is challenging.[2–9] Such reagents can be used to map ligand binding sites on proteins, DNA binding sites on proteins, and to explore protein–protein interactions. Protein sequencing studies,[10] in addition, can utilize such reagents to produce smaller fragments that are more amenable for sequencing. In addition to structural applications, such studies also improve our understanding of the molecular basis for recognition of small molecules by enzymes/proteins.

Several strategies are developed to induce protein cleavage by chemical reagents at selected sites.[2–9,11] Using metal complexes, reactive intermediates were generated for peptide bond cleavage at selected sites on proteins. Metal complexes were directed to specific sites in proteins by using affinity ligands. Peptide bond cleavage was achieved at the binding site by treating the protein bound metal chelate with a reducing agent and H_2O_2. Direct attachment of the metal complex to specific side chains in the protein is another strategy that has been proven to be successful for directed protein cleavage.[4,12,13] These approaches are complemented by photochemical methods described below.

Photocleavage of proteins by small, designed organic molecules is being developed in our laboratory.[14,15] Photoreactive chromophores are attached to short peptides to induce photocleavage of proteins at the probe binding site. The resulting peptides are amenable to sequencing, an important factor for biochemical applications. BSA, for example, was cleaved by Py-Phe between Leu 346 and Arg 347 and lysozyme undergoes site specific photocleavage between Trp 108 and Val 109. Quantum yields for the photocleavage varied from 0.26 for lysozyme to 0.0021 for BSA. Py-Phe provided the first example of site specific photocleavage of a protein by an organic molecule. Flash photolysis studies indicated that the photocleavage involves the pyrene cation radical.

Current experiments are designed to examine the effect of selected amino acid residues on the photocleavage efficiency/specificity. The decrease in the efficiency of the

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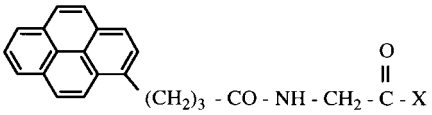

Chart 1. Structures of pyrenyl peptides: Py-Gly-X, where X=L-phenylalanine (Py-Gly-Phe), L-tyrosine (Py-Gly-Tyr), L-tryptophan (Py-Gly-Trp), or L-histidine (Py-Gly-His).

photoreaction from lysozyme (0.25) to BSA (0.02) is not due to decreases in the affinity of the probe. On the contrary, the binding constants of the probes are higher with BSA ($K_b > 10^6\ M^{-1}$) than with lysozyme ($K_b < 10^6\ M^{-1}$).[14,15] Local environment surrounding the probe is expected to have a substantial impact on the probe photoreactivity with the protein. Fluorescence yields of the protein bound probes, for example, are often less than the free probes, suggesting quenching of probe singlet excited state by amino acid residues at the binding site. The Py-Phe cleavage site (in both lysozyme and BSA) was lined with hydrophobic aromatic residues that may react with the probe singlet excited state or quench the reactive intermediate, pyrene cation radical, and interfere with the photocleavage mechanism.

The decrease in the cleavage efficiency in case of BSA, therefore, is suspected to be due to the quenching of Py^* or the cation radical by residues present at the binding site leading to dead-end products, or deactivation of the reactive intermediates. To test this hypothesis, we have explored the role of specific amino acid residues in the photocleavage mechanism by directly attaching such residues to the probe side chain and by examining their protein cleavage properties. The photoreactivities of amino acid conjugates of Phe, Tyr, Trp and His residues, in this context, are reported here (Chart 1). The pyrenyl chromophore is attached to these residues via a single Gly residue to moderate the intramolecular reaction, if any, between these residues and the Py^*. Pyrenyl probes containing short peptides Py-Gly-X (X=Phe, Tyr, Trp, and His) (Chart 1) are synthesized and their protein binding/photocleavage properties are reported here.

Materials and Methods

Lysozyme (MW=14,300), BSA (MW=66,267) are from Sigma Chemical Co., and the protein solutions were prepared by dissolving the appropriate amount of the protein in 50 mM Tris–HCl buffer, pH 7.0. All solutions are prepared fresh and used on the same day. The absorption spectra were recorded on a Hewlett–Packard Model 8453 diode-array spectrophotometer and calibration graphs have been constructed using Beer's law. Probe concentrations in our experiments were restricted to the linear region of the calibration graph. The molar extinction coefficients of the pyrenyl probes are 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 spectrometer interfaced with an Apple Macintosh computer using software developed in our laboratory. No excimer emission was observed from the peptide probes in the 0–50 μM concentration range. The absorption and fluorescence titrations with the proteins are performed by keeping the concentration of the probe constant while varying the protein concentration. The pyrenyl probe was excited at 345 nm, and the fluorescence intensity has been monitored at 377 nm as a function of protein concentration.

Synthesis of the peptide probes

Py-Gly-X (X=Phe, Tyr, Trp, and His) were synthesized by the N-terminal extension procedure using solid phase synthetic methods.[16] The first residue (N-blocked) was anchored to the solid phase via the carboxyl function. The N-terminal extension was carried out with the desired sequence of *N*-protected amino acid residues, and 4-(1-pyrenyl)butyric acid was used as the last residue in the synthesis. The peptide product was cleaved off the column, and the peptides were purified by HPLC (C-18 column) using a continuous solvent gradient from 100% water to 100% acetonitrile (both solvents contained 0.1% trifluoroacetic acid). The product solution was evaporated under vacuum and the residue was recovered as pure product. Py-Phe was synthesized as reported earlier.[14] Absorption maxima: 313, 326, and 343 nm; Fluorescence maxima: 377, 396, and 417 nm (345 nm excitation).

Photochemical protein cleavage

The protein photocleavage was carried out in 50 mM Tris–HCl buffer, pH 7.0. The protein solution (15 μM), containing Py-Gly-X (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 attached to a PTI model A1010 monochromator. UV cut-off filter (WG-345; 78%T at 344 nm) was used to remove stray UV light. Samples were withdrawn periodically to test the progress of the photoreaction. Irradiated samples were evaporated under vacuum for gel electrophoresis experiments.

SDS-Polyacrylamide gel electrophoresis and sequencing

SDS-PAGE Experiments were performed following literature methods with minor modifications.[17] Loading buffer (24 μl) (containing SDS (7% w/v), glycerol (4% w/v), Tris–HCl (50 mM), mercaptoethanol (2% v/v), and Bromophenol blue (0.01% w/v, adjusted to pH 6.8 with HCl)) was added to dry protein samples and the samples (8 μl) were heated for 3 min before loading onto the gel. While 12% polyacrylamide gels were used for lysozyme samples, 8% polyacrylamide gels gave good separations of BSA samples. The gels were run by applying 60 V until bromophenol blue passed through the stacking gel and the voltage was then increased to 110 V. The gels were run for 2.5 h for lysozyme or 1.5 h for BSA samples.

Flash photolysis studies

Samples were excited with a Spectra-Physics GCR-100 Nd-YAG laser or a continuum Surelite I laser (8 ns FWHM, 355 nm) and the signals were observed in the absorption mode using a transient digitizer, as described earlier.[18] The kinetic traces were monitored at specific wavelengths

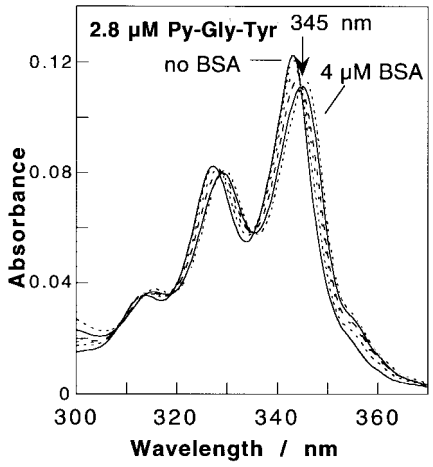

Figure 1. Absorption spectra of Py-Gly-Tyr (2.8 μM) with increasing concentrations of BSA (0–4 μM).

and the absorption spectra at various delay times were reconstructed from these traces.

Results

The pyrenyl peptides Py-Gly-X (X=Phe, Tyr, Trp, and His) show significant affinities for lysozyme (and BSA) and the binding constants with BSA vary as a function of X. The photoreactivities of the probes with both the proteins depended on the nature of the aromatic residue present in the probe side chain. The spectral changes and the photocleavage properties of the pyrenyl probes bound to BSA and lysozyme are reported here.

Absorption titrations

The pyrenyl chromophore responds to the changes in its environment when the probe solutions are exposed to BSA or lysozyme. The absorption spectra of Py-Gly-Tyr (2.8 μM) with increasing concentrations of BSA (0–4.0 μM) are shown in Fig. 1. The pyrenyl vibronic bands are red shifted by 3 nm (when compared to that of the free probe) with isosbestic points at 329, 336, and 345 nm. These

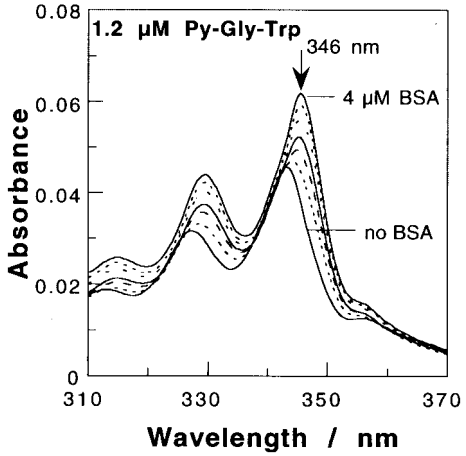

Figure 2. Absorption spectra of Py-Gly-Trp (1.2 μM) with increasing concentrations of BSA (0–4 μM).

shifts are also accompanied by weak hypochromism (10%). These red shifts and hypochromism parallel the results observed with Py-Phe/BSA and indicate binding of the pyrenyl probe to hydrophobic sites on the protein. Similarly, the Py-Gly-His absorption spectrum is red shifted by 2 nm, upon binding to BSA, with isosbestic points at 328, 337, and 344 nm (data not shown). The probe absorption spectra provide a convenient handle to monitor protein–ligand interactions. The observed spectral changes depended on the nature of the aromatic residue present in the probe side chain as well as the protein used for the studies.

Titration of Py-Gly-Trp with BSA resulted in contrasting results. The absorbance of Py-Gly-Trp (1.2 μM) increases with BSA concentration (0–4.0 μM) as shown in Fig. 2. The extinction coefficient of Py-Gly-Trp at 346 nm, for example, increased by 33% in the presence of 4.0 μM BSA when compared to the extinction coefficient of the free probe. The spectra are also red shifted in the presence of BSA and indicate hydrophobic environment similar to the surroundings of Py-Gly-Tyr or Py-Gly-His bound to BSA. Hyperchromism observed here is in contrast to the hypochromism observed with Py-Phe or Py-Gly-Phe.[15] These changes provide a strong evidence that the side chain of the probe plays an important role in the binding interaction, and perhaps in determining the location of the probe binding site on the protein. Similar hypochromism (but no peak shifts) was observed when Py-Phe binds to lysozyme.[14] The spectral differences noted here can be attributed to the nature of amino acids lining the probe binding site. Location of a charged residue, for example, at the binding cavity can stabilize or destabilize the transition dipole and change the spectral properties depending on the location of the charge with respect to the probe. Excitonic interactions between the probe and aromatic residues of the protein, on the other hand, may increase or decrease the extinction coefficient depending on the geometry and nature of the complex.[19] In any event, the spectral changes provide a strong evidence for the binding of the probes to the protein.

The absorption spectra are analyzed using the Scatchard equation (Eq. (1))[20] to estimate the binding constants of the probes. In Eq. (1), r is the ratio C_b/[protein] where C_b is the concentration of bound probe. The binding constant K_b is estimated by plotting the ratio of the binding density (r) to the concentration of the free probe (C_f) as a function of r. The absorbance changes at several wavelengths have been used to construct the binding isotherms for each wavelength and the binding constants for a given probe varied within ±5%. The binding constants depend on the nature of the aromatic residue present in the side chain (Table 1), and K_b decreases with increase in the hydrophilicity of the probe side chain by two orders of magnitude. In contrast, the binding constants of Py-$(Gly)_n$-Phe analogs (n=0, 1, 2) with BSA were nearly the same.

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

Fluorescence studies

The pyrenyl fluorescence provides another sensitive handle to examine the interaction of the probes with proteins. Py-Phe fluorescence, for example, was quenched by BSA,[15] in

Table 1. The binding constants (K_b, M^{-1}) and other binding properties of the pyrenyl probes

Probe	Py-Phe	Py-Gly-Phe	Py-Gly-Tyr	Py-Gly-Trp	Py-Gly-His
K_b/M (with BSA)	6.5×10^7	1.5×10^6	4.3×10^6	6.7×10^5	3.6×10^5
Isosbestic points (nm)	328, 336, 345	328, 336, 345	329, 336, 345	–	328, 337, 344
Hypochromism (−%) or hyperchromism (+%)	−17	−10	−10	+33	−3
Excimer Emission	Yes	–	No	No	No
Photocleavage	Yes	Yes	No	No	Yes

contrast, Py-Gly-Tyr (3 μM) fluorescence increases by the addition of BSA (0.4, 1.2, 1.6, 2.4 μM). No shifts in the spectral maxima (345 nm excitation, Fig. 3) are noted at these protein concentrations. The lack of changes in the pyrenyl fluorescence is somewhat surprising since this chromophore is extensively used to investigate microenvironments. The polarity of protein backbone, helix dipoles, and polar functions of the protein surrounding the chromophore are to be taken into account to interpret the changes in the photophysical properties. No new bands at longer wavelengths, characteristic of pyrene excimer emission, are observed. Excimers are excited state dimers formed between the ground state and excited state of the same molecule. New, broad, featureless emission bands were observed when Py-Phe, or Py-Gly-Gly-Phe bind to BSA, suggesting excimer emission in these cases.[15] Addition of BSA to Py-Gly-His (3 μM) results in very weak quenching of Py-Gly-His fluorescence (data not shown), and no shifts in peak positions or no new bands are noted. The absorption and fluorescence spectral studies clearly confirm the differences in the photophysical properties of the probes bound to BSA.

Titration of lysozyme (0, 2, 4, 6, 8, 10 μM) into solutions containing Py-Gly-X analogs indicates only a weak quenching of pyrenyl fluorescence and these observations are similar to those obtained with Py-Phe. No excimer or exciplex emission was detected with any of these probes bound to lysozyme, suggesting that binding occurs such that each probe molecule is isolated from the others. These data are consistent with the absorption and fluorescence properties of

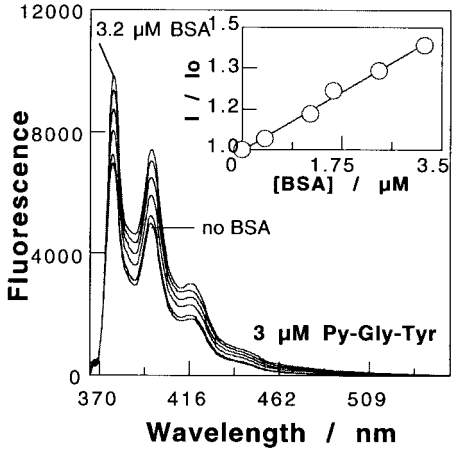

Figure 3. Fluorescence spectra of Py-Gly-Tyr (3 μM) with increasing concentrations of BSA (0–3.2 μM). Inset: Plot of I/I_o as a function of [BSA]. Py-Gly-Tyr fluorescence increases linearly with increase of BSA concentration.

Py-$(Gly)_n$-Phe analogs bound to lysozyme[15] and indicate a common binding site on lysozyme for these pyrenyl peptides. The differences in the properties of the protein-bound pyrenyl probes are further examined in protein cleavage studies.

Photocleavage of proteins

The probe-protein mixtures are irradiated at 344 nm in the presence of an electron acceptor, Co(III)hexammine (CoHA), and the protein photocleavage was monitored in gel electrophoresis experiments under denaturing conditions. The pyrene cation radical generated by electron transfer quenching of pyrenyl excited state by CoHA is expected to initiate protein cleavage at the probe binding site. The specificity and efficiency of photocleavage of BSA by Py-Gly-X probes are dramatically lower when compared to those of Py-Phe (Fig. 4). BSA was cleaved in the presence of Py-Phe (lane 2) resulting in just two fragments (M.Wt. of 41 and 28 kDa). Weak but reproducible cleavage was also observed with Py-Gly-His (lane 6) and these product bands resemble those with Py-Phe. The product yields with Py-Gly-His, however, are much lower than in case of Py-Phe. No distinct product bands are seen with the Tyr or Trp analogs of Py-Gly-X despite their high affinities for lysozyme and BSA. Current results, therefore, imply that the probe side chains play an important role in the protein photocleavage.

Photocleavage results observed with lysozyme by Py-Gly-X analogs is consistent with the above results (with BSA). The nature of the amino acid residues present in the linker control the photoreactivity of the probe and lysozyme photocleavage is shown in Fig. 5. A new product band is evident in lanes 2, 3, and 6. Py-Gly-Phe (lane 3) and Py-Gly-His (lane 6) cleave lysozyme with yields that are compar-

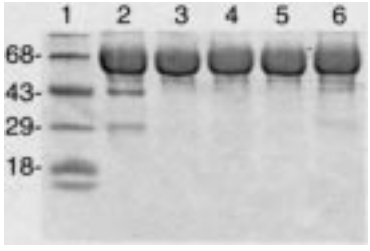

Figure 4. Gel electrophoresis pattern of BSA: lane 1, molecular weight standard (indicated in kDa); lane 2, BSA (15 μM), Py-Phe (15 μM), and CoHA (1 mM); lane 3, BSA (15 μM), Py-Gly-Phe (15 μM), and CoHA (1 mM); lane 4, BSA (15 μM), Py-Gly-Tyr (15 μM), and CoHA (1 mM); lane 5, BSA (15 μM), Py-Gly-Trp (15 μM), and CoHA (1 mM); lane 6, BSA (15 μM), Py-Gly-His (15 μM), and CoHA (1 mM). All samples in lanes 2–6 were irradiated at 344 nm for 60 min.

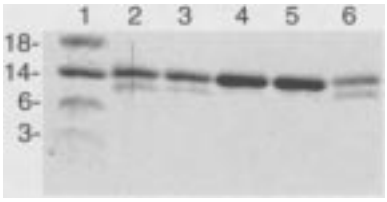

Figure 5. Gel electrophoresis pattern of lysozyme: lane 1, molecular weight standard (indicated in kDa); lane 2, lysozyme (15 μM), Py-Phe (15 μM), and CoHA (1 mM); lane 3, lysozyme (15 μM), Py-Gly-Phe (15 μM), and CoHA (1 mM); lane 4, lysozyme (15 μM), Py-Gly-Tyr (15 μM), and CoHA (1 mM); lane 5, lysozyme (15 μM), Py-Gly-Trp (15 μM), and CoHA (1 mM); lane 6, lysozyme (15 μM), Py-Gly-His (15 μM), and CoHA (1 mM). All samples in lanes 2–6 were irradiated at 344 nm for 20 min.

able to that of Py-Phe/lysozyme (lane 2). A second product band in lanes 2, 3, and 6 is often present, but too faint to see in Fig. 5. The major product observed with Py-Gly-Phe and Py-Gly-His is similar in molecular weight to that from Py-Phe. Irradiation of lysozyme, in contrast, in the presence of Py-Gly-Tyr or Py-Gly-Trp did not yield any fragmentation (lanes 4, 5). No protein cleavage was observed in the absence of the pyrenyl probe, CoHA, or light, indicating that these are essential for the protein photocleavage. The photoreactivity, thus, depends on the type of residues present in the probe side chain. Tyr and Trp, if present in the peptide probe, suppress the photocleavage. The reasons for the lack of photoreactivity of the Tyr and Trp analogs are investigated in flash photolysis studies.

Flash photolysis

Facile production of the $Py^{+\cdot}$ from Py-Phe, in the presence of CoHA, was demonstrated previously. The cation radical has a strong absorption around 460 nm with a half life of ~22 μs. The 460 nm transient was assigned based on its absorption spectrum, oxygen quenching rate constant, and reactivity towards electron donors.[14,21] In contrast, photoexcitation of Py-Gly-Tyr or Py-Gly-Trp (30 μM) in the presence of CoHA (3 mM) results in a minor transient that corresponds to the $Py^{+\cdot}$ (weak peak at ~460 nm, Fig. 6A) and a distinct absorption at 405 nm, assigned to the $Tyr^{\cdot}$. The $Py^{+\cdot}$ produced from Py-Gly-Tyr is short lived (Fig. 6B) and the transient absorption spectrum is weak. The half life,

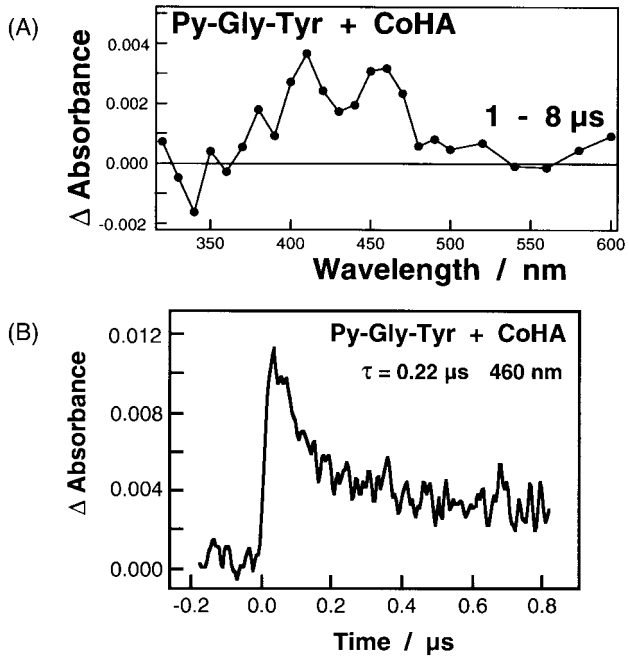

Figure 6. (A) Transient absorption spectrum of Py-Gly-Tyr/CoHA recorded (1–8 μs) after excitation. Bleaching at 345 nm is due to ground state depletion. (B) Decay of $Py^{+\cdot}$ monitored at 460 nm, upon excitation of Py-Gly-Tyr (30 μM)/CoHA (3 mM). The data are fitted to a single exponential decay with a rate constant of 4.4×10^{6} s^{-1}.

measured at 460 nm, is 220 ns (Fig. 6B), considerably shorter than that of $Py^{+\cdot}$ derived from Py-Phe (22 μs). Intramolecular quenching of $Py^{+\cdot}$ by Tyr could be responsible for this reduced lifetime and such quenching leads to $Tyr^{+\cdot}$. Loss of a proton from $Tyr^{+\cdot}$ will result in $Tyr^{\cdot}$. The weak absorption peak at 405 nm evident in the transient spectrum (Fig. 6A) could be due to the tyrosyl radical and this radical is known to absorb at 405 nm.[22] In contrast, laser excitation of Py-Gly-Tyr in the presence of BSA/CoHA, produced a transient with a strong absorption peak at 420 nm, characteristic of the pyrene triplet (Fig. 7A). The intensities of the peaks in these spectra can be compared based on the bleaching of the ground state observed at 345 nm (Fig. 6A and 7A). The lifetime of the 420 nm transient is ~31 μs comparable to the reported value for pyrene triplet.[14] The protein matrix surrounding the probe quenches the initially

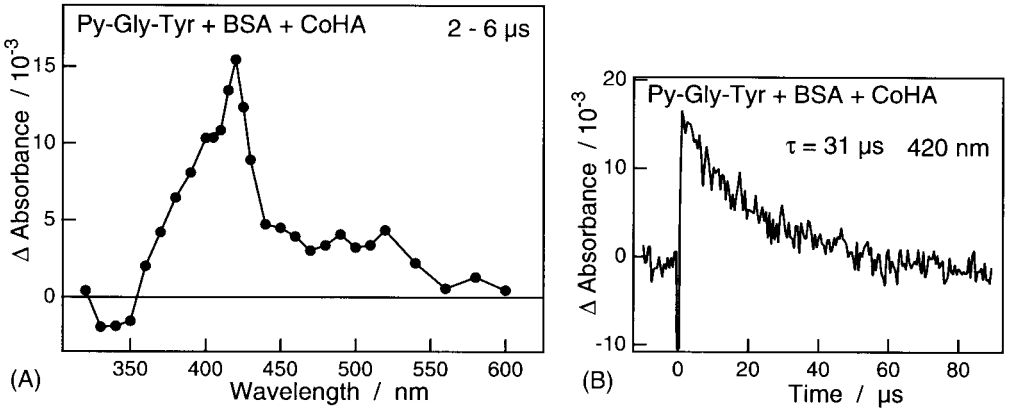

Figure 7. (A) Transient absorption spectrum of Py-Gly-Tyr (30 μM) in the presence of BSA (30 μM) and CoHA (3 mM). The strong peak at 420 nm is due to $^{3}Py^{*}$ and absorbance at 460 nm is very weak. (B) Transient decay of Py-Gly-Tyr (30 μM)+BSA (30 μM)+CoHA (3 mM) at 420 nm with a lifetime of 31 μs.

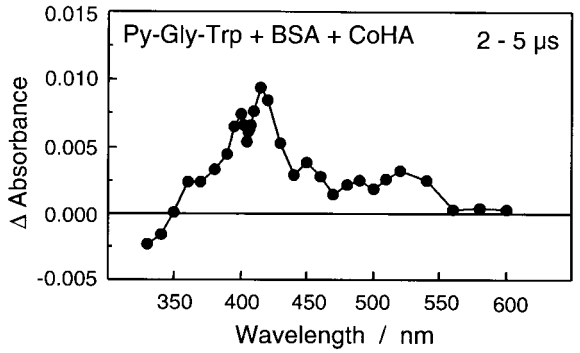

Figure 8. Transient absorption spectrum of Py-Gly-Trp (30 μM), in the presence of BSA (30 μM) and CoHA (3 mM). Peaks at 420, 405, and 520 nm assigned to $^3Py^*$, $Tyr^{\cdot}$, and $Trp^{\cdot}$, respectively.

produced cation radical or the precursor singlet excited state and enhanced triplet formation was noted. Enhanced fluorescence of Py-Gly-Tyr/BSA suggests that the singlet quenching pathway is less likely and the enhanced triplet formation is a product of the electron transfer quenching of the pyrenyl cation radical by the protein matrix. Enhanced triplet formation was also noted in the case of Py-Phe bound to BSA.[14]

Direct excitation of Py-Gly-Trp in the absence of protein or CoHA resulted in a strong absorption at 420 nm, indicating enhanced triplet formation (τ=24 μs, data not shown). Excitation of Py-Gly-Trp in the presence of BSA (30 μM)/CoHA (3 mM) resulted in strong transient absorption at 420 nm and additional peaks at 405, and 520 nm are also observed (Fig. 8). No absorption peaks characteristic of the pyrene cation radical are evident and the weak absorption at 520 nm characteristic of $Trp^{\cdot}$[23] indicate the rapid quenching of the $Py^{+\cdot}$ by the Trp residue. These observations are consistent with the fact that no protein cleavage occurs under these conditions. Current results are in contrast to the strong transient observed with Py-Phe/BSA/CoHA.[14] The flash photolysis data, thus, corroborate with the photocleavage experiments and suggest rapid quenching of the intermediate $Py^{+\cdot}$ by Tyr and Trp residues.

The subtle role played by the probe side chain on the binding affinity and the photocleavage of the pyrenyl probes is demonstrated here for the first time. The Tyr and Trp residues, if present in the probe, suppress the cleavage chemistry. Identifying the specific residues that interfere with the cleavage chemistry will be useful in the design of future peptide probes for protein cleavage.

Discussion

Absorption and fluorescence spectral data clearly indicate the binding of pyrenyl peptides to BSA and lysozyme. In case of BSA the absorption spectra are red shifted for all the pyrenyl peptides. In case of Py-Phe, Py-Gly-Phe, and Py-Gly-Tyr hypochromism is observed (with BSA), whereas hyperchromism was noted with Py-Gly-Trp. This observation is consistent with the binding of the probes to a generic hydrophobic site but the actual location of the pyrenyl residue at this site depends on the side chain structure. These probe-dependent spectral changes indicate the interaction of the chromophore with specific residues at the probe binding site.

In case of lysozyme, however, all the above probes exhibit hyperchromism with no shifts in peak positions, suggesting that all the probes may be binding at the same site on lysozyme or that the differences in the binding sites of the probes are too subtle to impact on the properties of the pyrenyl chromophore. Photocleavage results are consistent with the former possibility.

The variation in the binding constants over 2 orders of magnitude (with BSA) from Py-Phe to Py-Gly-His, clearly indicate decreases in the affinities with reduced hydrophobicity of the probe side chain. This result is in contrast with the fact that Py-$(Gly)_n$-Phe analogs have essentially the same affinity for BSA. Binding is weaker when the overall hydrophobicity of the probe is lowered by substitution with hydrophilic residues. The binding site for these probes is likely to be similar to that of Py-Phe, in domain II subdomain c, the known fatty acid binding site on BSA.[24] The photocleavage, and sequencing results with Py-Phe are consistent with the binding of the peptide probes at this site and the fragments observed in the current studies are similar to those obtained with Py-Phe/BSA.

Fluorescence studies suggest that Py-Gly-X binding site in BSA is buried in the protein matrix, away from the aqueous phase, consistent with the binding site discussed above. Enhanced fluorescence from Py-Gly-Tyr/BSA is perhaps due to hydrophobic burial as indicated in the absorption spectra. Intramolecular quenching of Py^* by Tyr and inhibition of such quenching upon binding to the protein is not ruled out, as an alternate mechanism. Enhanced emission was also observed when Py-Phe-Gly-Gly binds to BSA, where no such intramolecular mechanism is expected.[15] In case of lysozyme, the minor changes observed in the fluorescence spectra indicate that the probe bound to lysozyme is well exposed to the aqueous medium, consistent with the absorption data, and also in agreement with the known binding site of Py-Phe on lysozyme,[14] near residues 108 and 109, well exposed to the aqueous phase.

The effect of the probe side chain on the protein cleavage is strongly demonstrated in photocleavage and flash photolysis experiments. While no photocleavage of BSA or lysozyme is indicated with Py-Gly-Tyr and Py-Gly-Trp, significant activity is seen with Py-Gly-Phe and Py-Gly-His. Photocleavage yields are much greater with lyoszyme, in general, than with BSA. This is because the probes bound to lysozyme are exposed to the solvent and their excited states can be rapidly quenched by CoHA to produce the reactive cation radical. In contrast, the probes are buried in a hydrophobic cavity in BSA making them less accessible to CoHA. Even in case of BSA where the probes are less accessible, Py-Phe, and Py-Gly-His show considerable activity while the Tyr and Trp analogs are not reactive. These variations in the cleavage yield can be explained using flash photolysis data. Intramolecular quenching of the $Py^{+\cdot}$ by Tyr or Trp residues leads to the deactivation of the key intermediate in competition with protein photocleavage. The lifetime of the $Py^{+\cdot}$ decreases from 22 μs in case of Py-Phe to 220 ns with

Py-Gly-Tyr, consistent with intramolecular quenching mechanism. The Tyr and Trp analogs are not active even with the more reactive lysozyme, consistent with the conclusion that Tyr and Trp residues interfere with the cleavage chemistry.

Therefore, Tyr and Trp residues are detrimental to the photoreactivities of these probes, while Phe, Gly, and His residues permit the cleavage chemistry to a significant extent. These insights are useful in our future design of the probes for protein photocleavage.

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