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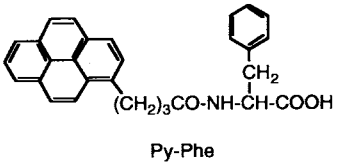
Site-Specific Photocleavage of Proteins**

Challa V. Kumar* and Apinya Buranaprapuk

Dedicated to Professor Nicholas J. Turro on the occasion of his 59th birthday

Reagents that cleave proteins with high specificity under thermal or photochemical conditions could be useful for structure–activity studies of proteins, investigation of protein structural domains, design of new therapeutic agents, and conversion of large proteins into smaller fragments that are more amenable for sequencing.[1] The proliferation of chemical nucleases in DNA-cleavage studies is an example of how reagents based on small molecules can be of use in molecular biology.[2, 3] Analogously, chemical proteases are useful for the manipulation of proteins.[1, 4, 5] Two approaches for the design of chemical proteases can be distinguished: 1) use of affinity-based reagents, and 2) covalent linking of redox-active metal complexes to specific residues of the protein with subsequent activation of the metal to achieve protein cleavage. We describe here a new reagent for the photocleavage of proteins that has a) high affinity for hydrophobic sites of proteins, b) strong absorption bands in the near-visible region, c) a long-lived singlet excited state (which is convenient for photoreactions), and d) a chromophore that has been extensively used to probe microenvironments. Use of light as a reagent in protein-cleavage reactions provides strict control of the initiation and termination of the reaction. Vanadate was used as a photoprobe for phosphate-binding sites of proteins.[6] Vanadate has a low extinction coefficient (>320 nm),[6] and high concentrations of vanadate are needed to capture a significant fraction of the incident light. The presence of several vanadium species in vanadate solutions makes it difficult to identify the photochemically active species,[6c] and vanadate induces photocleavage at many sites other than serine residues.[6d] We now observed that Py-Phe (Scheme 1) complements vanadate photochemistry and that Py-Phe induces site-specific photocleavage of bovine serum albumin (BSA) and chicken-egg lysozyme under mild conditions.[7]

The design of Py-Phe was prompted by earlier studies in our laboratory with bifunctional probes consisting of hydrophobic and hydrophilic groups separated by a linker.[8–10] Bifunctional probes seek out binding sites on proteins that have an arrangement of hydrophobic and hydrophilic microenvironments with complementary interactions. Covalent linking of 4-(1-pyrenyl)-butyric acid with L-phenylalanine resulted in fluorescent Py-Phe (Scheme 1).[11] The hydrophilic carboxy functionality of Py-Phe and the amide linkage improve the aqueous solubility of the hydrophobic pyrene moiety of Py-Phe. The hydrophilic functionalities may also participate in electrostatic and hydrogen-bonding interactions. The size of the separation between the hydrophobic and hydrophilic moieties (the length of the linker) plays an important role in probe binding to proteins. Probe affinity for proteins, for example, increases with increasing linker length as well as with the size of the hydrophobic moiety.[8] The long-lived singlet excited state of the pyrenyl chromophore ($t_{1/2}$ > 100 ns), when coupled with the redox activity of amminecobalt(III) complexes, results in site-specific protein cleavage.



Py-Phe

Scheme 1. Structure of the bifunctional probe Py-Phe, which is obtained by linking 4-(1-pyrenyl)butyric acid with L-phenylalanine.

Studies show that Py-Phe binds to proteins. For example, the electronic absorption spectrum of Py-Phe (3 μM) is red-shifted upon binding to BSA (Figure 1). The intensities of Py-Phe absorption bands decrease with increased protein concentration;

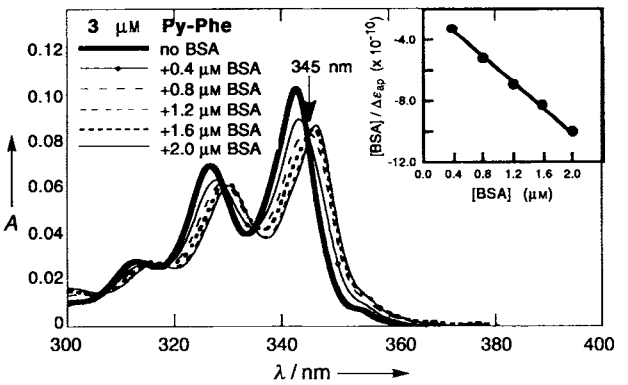


Figure 1. Absorption spectra of Py-Phe (3 μM) with and without BSA. The spectra are red-shifted by 4 nm with an isosbestic point at 345 nm. A plot of the quotient of the BSA concentration and $\Delta\varepsilon_{ap}$ versus the BSA concentration is shown at the top right corner; the estimated binding constant for the binding of Py-Phe to BSA is $(2.8 \pm 0.5) \times 10^6\ M^{-1}$. The binding constant for the binding of Py-Phe to lysozyme is $(7 \pm 0.5) \times 10^5\ M^{-1}$. A = absorbance.

the isosbestic point is at 345 nm. The red shift and hypochromicity indicate a hydrophobic environment around the pyrenyl chromophore.[8] The binding plot[12] derived from the absorption titration data (inset of Figure 1) provided a binding constant of $(2.8 \pm 0.5) \times 10^6\ M^{-1}$. Binding of Py-Phe to BSA was also confirmed by circular dichroism (CD) and fluorescence spectra. New positive CD bands appeared at 330 and 345 nm when BSA (50 μM) was added to Py-Phe (50 μM), whereas the free probe had negative CD bands at 336, 351, and 360 nm. Binding of Py-Phe to BSA increases the fluorescence lifetime of Py-Phe from 106 ns (free probe, air-saturated buffer) to 125 ns (1:1 ratio of Py-Phe to BSA). Fluorescence-quenching studies indicate that Py-Phe bound to BSA is well protected from the

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aqueous medium. The Stern–Volmer quenching constants obtained with CoHA (= $[Co(NH_3)_6]^{3+}$) as the quencher decrease from 1.9 mM^{-1} (free probe) to 0.25 mM^{-1} (bound to BSA). Taken together, these spectroscopic studies indicate that the probe binds to the protein through its pyrenyl moiety, which is protected from the aqueous medium by the surrounding protein matrix.

Photocleavage of BSA was achieved by irradiating into the absorption bands of the pyrenyl chromophore at 344 nm in the presence of CoHA (1, 2, and 10 mM) as an electron acceptor. Progress of the protein photocleavage was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE; 6% acrylamide, 0.4% bis(acrylamide), 13% glycerol, pH 8.4).[13] Two new bands for BSA fragments with molecular weights of 41 and 28 kDa appear in the gel (Figure 2), illustrating the high degree of specificity in cleavage. If the photocleavage were random, one would observe a smear in these lanes. No photocleavage is observed in the absence of either of the two reagents or light (Figure 2, bottom, lanes 3 and 8–10). Irradiation of BSA and CoHA without Py-Phe does not yield any fragmentation (lane 10). Similarly, irradiation of BSA in the presence or absence of Py-Phe without CoHA does not result in fragmentation (lanes 9 and 8, respectively). Py-Phe, CoHA, and light are essential for the observed photocleavage. Photocleavage with Py-Phe is also observed for lysozyme (two fragments of lower molecular weight (11 kDa, 3.5 kDa) appear), myoglobin, and carboxypeptidase, demonstrating the general nature of the methodology.

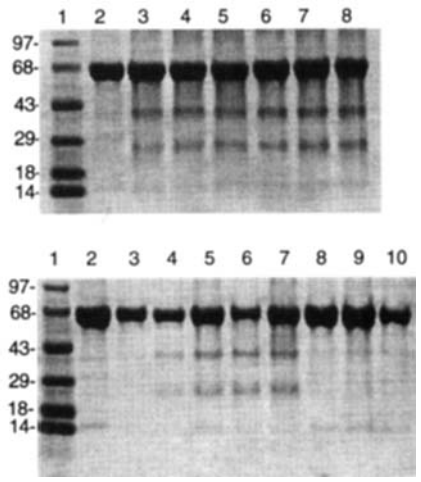

Figure 2A. Top: the gel-electrophoresis pattern of BSA under various conditions: lane 1, molecular weight standard for the mass values given on the left; lanes 2–8 contained BSA (15 μM) and Py-Phe (15 μM); lanes 3–5 also contained CoHA (1 mM); lanes 6–8 also contained CoHA (2 mM). Samples in lanes 3–5 were irradiated at 344 nm for 20, 40, and 60 min, respectively. Samples in lanes 6–8 were irradiated for 20, 40, and 60 min, respectively. The samples were run from top to bottom. Bottom: the gel-electrophoresis pattern of BSA under various conditions: lane 1, molecular weight standard for the mass values given on the left; lane 2 contained BSA (15 μM); lanes 3–7 contained BSA (15 μM), CoHA (10 mM), and Py-Phe (15 μM), and the sample was irradiated for 0, 10, 20, 30, and 60 min, respectively; lane 8 contained only BSA (15 μM), and the sample was irradiated for 30 min; lane 9 contained BSA (15 μM) and Py-Phe (15 μM), and the sample was irradiated for 30 min; lane 10 contained BSA (15 μM) and CoHA (10 mM), and the sample was irradiated for 30 min.

Use of Co^{III} complexes other than CoHA—such as chloropentamminecobalt(III) chloride, $[Co(bpy)]_3^{3+}$, or sepulchratecobalt(III) chloride—also results in the same fragments for the cleavage of BSA with Py-Phe. The cleavage most likely occurs at the pyrenyl-binding site rather than at the metal-binding site. Singlet oxygen is not involved in these reactions, as degassing of the reaction mixture does not alter or reduce photoproduct formation. Addition of sodium azide, a known singlet-oxygen quencher, does not inhibit the photoreaction. Irradiation for longer than 20 min enhances the formation of photoproducts only marginally (maximum conversion of 21% for BSA and 44% for lysozyme). The pyrenyl radical cation, generated by electron transfer from the Py-Phe singlet excited state by CoHA, may be responsible for the photocleavage. The following support this view: Py-Phe fluorescence is quenched by CoHA at a diffusion-controlled rate (4.6×10^9 $dm^3 mol^{-1} s^{-1}$), amminecobalt(III) complexes are necessary for cleavage, Py-Phe is gradually consumed in the photoreaction, and $[Co^{II}(H_2O)]_6^{2+}$ is detected at the end of the photocleavage reaction. Fluorescence from protein-bound Py-Phe is quenched by CoHA at a slower rate than that with the free probe (K_{SV} is reduced by a factor of about 8 upon binding to BSA). When Py-Phe is omitted from the reaction mixture, irradiation does not lead to protein cleavage. The pyrenyl chromophore is important in the light-absorption process and in the subsequent photoreaction. The N-terminal sequencing of the photoproducts to identify the cleavage site was not successful, which suggests possible N-terminal blockage similar to those observed with other protein-cleavage protocols.[1a–c, 6] Experiments are in progress to identify the cleavage site and the mechanistic pathways responsible for photocleavage. The appearance of two sharp bands in the gels demonstrates a single cut in the peptide backbone with a high degree of specificity. The low diffusional mobility of the pyrenyl radical cation (compared to that of the hydroxy radical)[14] and the high affinity of Py-Phe for hydrophobic and hydrophilic microenvironments in proteins may be responsible for the observed high specificity.

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[11] L-Phenylalanine ethyl ester (0.4 g) and dicyclohexylcarbodiimide (0.4 g) were added 4-(1-pyrenyl)butyric acid (0.5 g) in acetonitrile (50 mL). The mixture was stirred at room temperature (4 h), and excess DCC deomposed. The solvent was removed under reduced pressure, and the reaction mixture subjected to acid hydrolysis (1 N HCl, 12 h, room temperature). Pure product was isolated after chromatography on silica gel. ^{1}H NMR (270 MHz, DMSO): δ = 8.06–8.34 (m, 9H), 7.2 (m, 5H), 4.5 (exchangeable NH, 1H), 2.0 (m, 1H), 2.1–2.5 (m, 6 aliphatic H); IR (KBr): $\tilde{\nu}$ = 3300 (OH), 1703 (acid C=O), 1640 cm^{-1} (amide C=O); the absorption maxiama at 313, 326, and 343 nm as well as the fluorescence maxima at 376, 396, and 417 nm (340 nm excitation) correspond to those of the pyrenyl chromophore. The circular-dichroism maxima are at 336, 351, and 360 nm.

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[13] The reaction mixture (0.1 mL) was irradiated at 344 nm for various lengths of time with a xenon lamp source and a monochromator (3.0×10^{-8} einsteins min^{-1} at 340 nm). Filters were used to remove stray UV light, and the band pass on the monochromator was adjusted to 10 nm. Irradiated solutions of the protein and probe were dried under reduced pressure, and the residue was redissolved in the sample buffer (0.024 mL) made of glycerol (1 mL), sodium dodecyl sulfate (3 mL, 10% aqueous solution), tris(hydroxymethyl)aminomethane hydrochloride (1.25 mL, 0.5 M), bromophenol blue (0.6 mL, 0.1% solution), and deionized distilled water (4.5 mL). The gels were run by applying a voltage of 60 V until the dye (Coomassie blue) passed through the stacking gel. The voltage was then increased to 110 V, and the gels were run for a total of 2 h; see H. Schagger, G. V. Jagow, *Anal. Biochem.* **1987**, *166*, 368.

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Design of Thermotropic Liquid Crystals with Micellar Cubic Mesophases: Amphiphilic *N*-(2,3-Dihydroxypropyl)benzamides**

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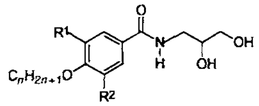
Cubic mesophases are common in surfactant/solvent and lipid/solvent systems and are ordered supermolecular arrangements with isotropic physical properties. They have attracted considerable interest because of their potential use in drug release systems, as templates for the preparation of mesoporous silicates, and as models for cell fusion processes.[1] Several different cubic mesophases are observed in these systems depending on the concentration of the surfactant.[2] They can occur as intermediate phases either between lamellar and hexagonal columnar phases (bicontinuous cubic phases, V-phases) or between the hexagonal columnar phases and the micellar solutions (discontinuous cubic phases, I-phases). The first type can be regarded as interwoven networks of branched columns, the second one consists of closed micelles arranged in a cubic lattice. The interface curvature between hydrophilic regions and lipophilic regions was recognized as the key factor determining the phase type. Depending on the sign of the interface curvature either normal phases (type 1; the interface curvature is directed away from the regions with stronger cohesive interactions) or inverted phases (type 2; the interface is curved toward the regions with stronger cohesive forces) can occur.

In contrast to the large number of lyotropic systems with cubic mesophases, relatively few thermotropic compounds with cubic phases are known.[3,4] In most cases, bicontinuous structures with body-centered cubic lattices (*Ia*3*d*, *Im*3*m*), which represent intermediate phases between columnar and smectic phases, have been proposed for these thermotropic cubic phases.[4] More recently different types of thermotropic cubic phases have been observed for amphiphilic carbohydrate derivatives.[5,6] For some of these compounds inverted micellar cubic phases with primitive cubic lattices (*Pm*3*n* or *P*43*n*) have been found.[6] Because this type of primitive cubic phase has never been observed in thermotropic systems, their more detailed investigation should be of special interest;[7] however, the transition temperatures of these carbohydrate derivatives are rather high such that their study is hampered by decomposition. In an effort to overcome these difficulties, we decided to decrease the number of attractive hydrogen bonds in the head group regions.

Here we describe the first stable model compounds for these carbohydrate derivatives. These are amphiphiles in which one, two, or even three lipophilic alkyl chains were grafted to a hydrophilic N-(2,3-dihydroxypropyl)benzamide group (**1**–**3**). As



1, $R^1 = R^2 = H$
2, $R^1 = C_nH_{2n+1}O$, $R^2 = H$
3, $R^1 = R^2 = C_nH_{2n+1}O$

in the case of related D-glucamides,[6] three different types of mesophases were found depending on the number of alkyl chains: compound **1** with only one dodecyloxy chain, $n = 12$, forms a smectic A_d phase, a hexagonal columnar phase (Col_{H2}) is found for the double chain compound **2**, and a cubic phase was detected for compound **3** with three dodecyloxy chains (Table 1). The X-ray diffraction pattern of this cubic phase can be indexed on the basis of a primitive cubic lattice (*Pm*3*n* or *P*43*n*; $a_{cub} = 7.45$ nm at $T = 90\,°C$; Table 2). Thus, simply by changing the number of lipophilic chains grafted to the aromatic linking units the supermolecular organization of these molecules can be changed from layerlike through columnar to cubic. In this respect the thermotropic mesomorphism of these compounds, which depends on the number of lipophilic chains, is analogous to the lyotropic mesomorphism of surfactant/solvent

Table 1. Transition temperatures *T* of the compounds **1**–**3**, $n = 12$, and lattice parameter *a* of their mesophases [a].

Compd.	R^1	R^2	T[°C] [a]	a[nm] (T[°C])
1, $n = 12$	H	H	cr 89 S_A 132 is	4.03 (85)
2, $n = 12$	$C_{12}H_{25}O$	H	cr 98 Col_{H2} 148 is	4.18 (105)
3, $n = 12$	$C_{12}H_{25}O$	$C_{12}H_{25}O$	cr 69 Cub_{I2} 126 is	7.45 (90)

[a] Abbreviations: cr = crystalline solid, S_A = smectic A-phase, Col_{H2} = hexagonal columnar mesophase, Cub_{I2} = inverted micellar cubic mesophase (space group *Pm*3*n* or *P*43*n*), is = isotropic liquid. The designation of the Col and Cub phases is given as subscripts using descriptors of lyotropic phases as proposed by Tiddy [2c]. The polar/lipophilic interfaces are curved in the direction of the polar regions (negative curvature) and thus represent type 2 phases (inverted phases).

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