1 Invited Feature Article

2 **Chiral Photochemical Scissors: Toward Site Specific Cleavage of Proteins with Light**

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4 Bobbi Stromer¹, Melissa Limbacher¹, Dhanya T. Jayaram², Sudarat Yenjai³, Ruma Chowdhury¹,
5 Apinya Buranaprapuk³, Danaboyina Ramaiah⁴, and Challa V. Kumar^{1,5*}

6

7 ¹*Department of Chemistry, 55 N. Eagleville Road, U-3060, University of Connecticut, Storrs,*
8 *Connecticut 06269-3060*

9 ²*CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST),*
10 *Thiruvananthapuram, 695019, India*

11 ³*Department of Chemistry, Srinakarinwirot University, Sukhumvit 23, Bangkok 10110, Thailand*

12 ⁴*CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, 785006, India*

13 ⁵*Department of Molecular and Cellular Biology, 91 N. Eagleville Road, U-3125, University of*
14 *Connecticut, Storrs Connecticut 06269-3125*

15 **Corresponding Author: Challa Vijaya Kumar, challa.kumar@uconn.edu*

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23 Table of Contents

24	1. Introduction	4
25	2. Rational design of the protein photocleavage reagents.....	5
26	3. Proteins examined	7
27	4. Protein photocleavage reagents:	8
28	4.1. Amino acid or peptide derivatives:.....	9
29	4.1.1 Photophysical Properties	11
30	4.1.2 Photochemical Studies	24
31	4.1.3 Computational Modeling	30
32	4.1.4 Flash Photolysis Studies and the Mechanism	33
33	4.2. Avidin-based Pyrenyl Probes	35
34	4.2.1 Synthesis and Characterization of Py-Biotin	36
35	4.2.2 Photophysical studies of Py-Biotin with Avidin	36
36	4.2.3 Computational Modeling	38
37	4.3. Squaraine Dyes	39
38	4.3.1 Photophysical studies	39
39	4.3.2 Photochemical Properties	44
40	5. Contributions of Others to the Field of Protein Photocleavage	45
41	6. Conclusions	46
42	7. Acknowledgments	48

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47 **Abstract:**

48 A powerful strategy to photocleave the protein backbone at a single site, developed over nearly two
49 decades, is described here. The remarkable ability to target one or even a couple of sites on a large
50 protein with a small molecule, under photochemical control, is a considerable challenge. Systematic
51 modification of the structure of the photoreagent provides valuable information on the binding site
52 recognition as well as the mechanism of the photocleavage reaction. Some factors that impact the
53 photocleavage reaction include the exact location of the probe binding site on the protein,
54 conformations of the bound probe, protein size, functional groups present on the probe that ~~can~~ interact
55 with the protein surroundings either in a favorable or unfavorable manner, overall charge on the
56 photoreagent, and photophysical/photochemical properties of the probe. The protein photocleavage
57 studies were preceded by detailed binding studies by a variety of spectroscopic methods. Methods as
58 simple as absorption and fluorescence spectroscopies or more sophisticated circular dichroism
59 spectroscopy were used. Conclusions that are most consistent with the binding data indicated a single
60 binding site on most proteins, irrespective of the probe or the protein, with only one exception noted so
61 far. Such high selectivity for targeting a binding site on a biomolecule by a small molecule is
62 remarkable. Photocleavage studies and laser flash photolysis studies were used to probe the
63 photochemical peptide bond cleavage reactions, which indicated the important role of aromatic cation
64 radicals in the mechanistic path. Our work focused mainly on few generic proteins serum albumin,
65 lysozyme, and avidin; but may be extended to other proteins with appropriate modifications. This
66 article strives to provide some guidance on such design strategies for other proteins or other
67 photoreagents. Molecular modeling studies clearly supported our conclusions and the methods
68 described here could be useful in a rational design of novel photoreagents to target desired sites on
69 proteins in future studies.

70

1. Introduction

Photochemical reactions provide exquisite control over the initiation and termination of chemical reactions, where the key reagent (light) vanishes before the workup of the reaction mixture. Therefore, light initiated protein-cleavage reaction methodology appealed to us, nearly two decades ago.¹ Reagents that cleave proteins upon activation with light were unknown at that time, and our group was the first to demonstrate that rationally designed organic molecules could cleave the peptide backbones of large proteins at a single site (site specific). The initial approach and the early methods have been modified to include inorganic photo reagents, which also demonstrated specificity or high selectivity for the photocleavage of proteins, sometimes in high yields, approaching 60%.² One interesting feature of these artificial photo proteases was that the newly produced N-terminus or the C-terminus (in some cases) of the product peptide fragments has been amenable to standard biochemical sequencing methods.^{3,7} Therefore, these reactions provided a useful tool for making smaller fragments from larger proteins that can be sequenced. This article provides an account of these investigations, challenges, failures, and specific approaches as well as the current status of this area of exciting protein photochemistry.

Protein photocleavage is also an interesting tool to footprint ligand binding sites on large biological structures such as protein/DNA complexes or protein/protein complexes or enzymes bound to solid surfaces.^{3,45} Such very large biological/chemical structures are not readily amenable to current approaches of biochemical methodologies such as cryo TEM or protein mass spectrometry.^{2,3,6} Protein photocleavage studies can, therefore, provide valuable structural data to better understand the molecular basis for recognition in the solution phase, under ambient conditions. Structures of biomolecules in the solution phase are often more relevant to functional biological systems where bimolecular or multimeric structures are important. This understanding in liquid phase could be important to manipulate and control biological processes such as cell communication, cell adhesion, cell motion, gene expression, and the fight against antibiotic resistance.⁷

An unexpected application that emerged out of our photochemical studies is the design, construction and testing of artificial light antenna systems based on ordinary proteins.⁸ This strategy arose from the conclusion that organic ligands can be designed to bind to discrete sites on selected proteins, thus allowing us to assemble large numbers of chromophores in proteins at defined distances. This also allowed for the control of specific orientation of the chromophore and tuning of the affinities,

100 while suppressing chromophore aggregation and undesirable excited state annihilation or quenching.
101 These molecular assemblies made of dyes and proteins functioned as efficient visible light harvesting
102 antennas.⁹

103 2. Rational design of the protein photocleavage reagents

104 Site specific photocleavage (cleavage at a single site) of proteins can be achieved by directing
105 the reagent to a specific site on the target protein and subsequent photoactivation to cleave the peptide
106 backbone. Our experience showed that even moderate binding affinities and moderate photochemical
107 quantum yields are sufficient to achieve this goal. However, the design of the reagent is critical in
108 achieving binding at a particular site on the protein, which could endow specificity for the
109 photocleavage reaction. This unexpected outcome was due to the fact that the binding sites in proteins
110 are highly crowded for moderately sized photoreagents, and the excited state or the reactive
111 intermediate does not have necessary freedom to explore different parts of the binding site.

113 Scheme 1. A. Chiral protein scissors that bind to protein and cleave on irradiation at specific sites. B.
114 General strategy for photoreagent design. C. Pyrenyl photocleaving agent, Py-(L or D)-Phe. Scheme A
115 reproduced with permission from Langmuir.

117 The photoreagent design is, therefore, critical and should take into account structural features
118 of the photoreagent such as size, shape, H-bonding groups, dipoles, charged groups and chirality for
119 proper interaction with the binding site on the target protein (Scheme 1A, B). These features allow
120 discrimination of a given binding site by the reagent against other possible sites on the target protein
121 and could reduce the number of possible binding sites. Thermodynamically, this would drive binding
122 selectivity/specificity, and photoexcitation of the protein-bound reagent, under these conditions, to
123 produce fewer cleavage sites than if the binding occurs at multiple sites. Our goal in the early studies

124 was to bind the photoreagent to a single site on a given target protein, among multiple possible binding
125 sites. Modern computational modeling using powerful docking algorithms have made excellent
126 progress in this aspect, and docking studies matched well with the cleavage sites observed for a few
127 proteins and a handful of photocleavage reagents.² While this outcome is very satisfying and
128 supportive of the methodology described in this article, the design of a new reagent to cleave at a
129 desired site on a target protein is still a fundamental, unsolved problem.

130 Therefore, a qualitative approach based on the principle of ‘Complementarity’ has been
131 explored in this article. Our main assumption is that the ligand structure, shape, and interaction sites
132 should ‘Complement’ those at the binding site on the target protein to achieve the lowest free energy
133 bound state (Scheme 1). Our initial studies were directed to evaluate unique binding sites on common
134 proteins such as serum albumin, lysozyme, and avidin. Our ligand design included: (1) a hydrophobic
135 chromophore which is to be buried in the non-polar interior of a protein; (2) a hydrophilic anchor
136 group that would prefer to reside in the aqueous medium near the protein/water interface, and (3) a
137 suitable linker connecting the polar and nonpolar groups of the reagent so that the two regions can be
138 separated well enough to satisfy the requirements of the binding site (Scheme 1B).

139 These principles rose out of the observation that the interior of most water-soluble proteins are
140 fairly lipophilic while their exterior is strongly hydrophilic (oil drop model of a protein).¹⁰ With this
141 combination of contrasting interactions (a hydrophobic group and a hydrophilic group separated by a
142 linker) it would be possible to direct the photoreagent to specific sites on the protein at the
143 solvent/protein interface, with the most favorable binding site complementing the specific interactions
144 supported by the photoreagent. For example, a positively charged hydrophilic group on the reagent
145 would prefer to reside next to an anionic side chain at the binding site. Similarly, the hydrogen
146 bonding donor and acceptor sites of the binding site would complement that on the reagent, at optimal
147 distances so that the free energy of the system would be reduced. These multiple prerequisites enhance
148 the binding selectivity of the probe and hence, increase the number of these interactions, enhancing the
149 selectivity for binding. Highly selective binding could in turn improve selectivity for subsequent
150 protein photocleavage. This approach of ‘Complementarity’ has been very successful and provided a
151 powerful algorithm to direct ligands to a single site even on a very large protein that has multiple,
152 known, ligand binding sites.²

The next important step in the design was to include an appropriate chromophore which would absorb the photon of desired wavelength and initiate the photoreaction that would ultimately result in peptide bond cleavage at the reagent binding site. We tested both organic¹⁻⁷ as well as inorganic¹¹ chromophores to initiate the protein photocleavage reactions. For example, the pyrene chromophore served as the hydrophobic group to anchor the probe in the protein interior (Scheme 1C). A short peptide was chosen to be the linker connecting the chromophore to the polar/charged group (Scheme 1B,C). The peptide linker would complement interactions with the peptide chain of the protein and it is expected to promote photoreagent binding to the protein via hydrogen bonding interactions.

The use of a positively charged Co(III) metal complex was envisioned as the polar group ligated to the C-terminus of the peptide linker of the photoreagent, in the above design, but initial studies were done without ligation of the metal complex. Thus, C-terminus of the peptide linker itself served as the polar group, and a redox metal complex served to sensitize the photo oxidation of the otherwise unreactive pyrene excited state of the probe. For example, Co(III)hexamine (CoHA) and other Co(III) complexes are well known to quench pyrene excited state and produce the corresponding aryl radical cation.^{2,4,6} Our hypothesis was that the pyrene excited state has sufficient energy to sensitize the photo decomposition of Co(III)hexamine, and photo decomposition of CoHA is known to result in reactive NH₂ radical.¹² The central hypothesis was that the NH₂ radical would abstract a hydrogen atom from the peptide backbone and ultimately lead to peptide bond cleavage, in a manner similar to CoHA photocleavage of the sugar phosphate backbone of double helical DNA.¹³ However, at that time none of the related protein photocleavage chemistry by Co(III) complexes was known. Thus, these hypotheses were considered high-risk-high-dividend studies.

Use of photons for protein cleavage allows for strict control over the initiation and termination of the reaction, which facilitates mechanistic studies via time-resolved methods. These are green reaction conditions where one of the reagents (light) disappears and simplifies the reaction workup. The above interesting hypotheses were tested over the last two decades^{1-7,14,15,16} and lessons learned in this endeavor are enumerated below.

3. Proteins examined

Bovine Serum Albumin (BSA), avidin, and lysozyme are utilized in the experiments described below and extensions to other proteins are possible with appropriate modifications of the current methodology. BSA (Figure 1A) is a 65 kDa, major carrier protein of the circulatory system, and it

183 transports lipids, metal ions, amino acids, hormones, and peptides. Most drugs entering the blood
184 circulation encounter this protein, and therefore, BSA has been extensively studied for its interaction
185 with small organic molecules, peptides and hormones.¹⁷ BSA is a heart shaped protein made up of
186 three homologous domains with multiple, major binding sites that support ligand binding via a
187 multitude of hydrophobic, hydrogen bonding, and electrostatic interactions.^{18,19}

189 *Figure 1: Structures of A) Bovine serum albumin (BSA) (PDB ID: 3VO3)²⁰, B) Lysozyme (PDB ID:*
190 *1DPX)²¹, and C) Avidin (PDB ID: 2AVI)²² used for photocleavage studies in this article.*

191

192 Lysozyme (Figure 1B) is a small (14.3 kDa), water soluble enzyme present in numerous
193 biological fluids, including human tears. It is part of the innate immune system and it can hydrolyze
194 and break down bacterial cell walls, especially gram positive bacteria.²³ Lysozyme is the first enzyme
195 to be crystalized and have its structure determined by X-ray diffraction.²⁴ Avidin (Figure 1C) is a 60
196 kDa tetrameric glycoprotein that binds strongly to biotin with an affinity constant of 10^{15}M^{-1} , which is
197 nearly as strong as a weak covalent bond.²⁵ Avidin is made of four identical subunits, each of which
198 can bind one biotin molecule. Biotin binding site is within the core of the eight stranded, antiparallel
199 β -barrel that makes up each subunit. The strong binding of biotin is frequently exploited in avidin-
200 biotin technology for biological assays.²⁶

201 4. Protein photocleavage reagents:

202 A number of different classes of reagents were tested for protein photocleavage and these
203 included four major classes: (A) amino acid or peptide derivatives,¹⁻⁷ (B) avidin based reagents,⁵ (C)
204 squaraine derivatives,^{27,28} (D) uranyl ions¹⁴ and (E) coordination metal complexes.^{11,15,16} Among
205 these, only the first three systems are presented here, in that order. Design of each of these classes of
206 reagents, their interactions with target proteins, and their photocleavage reactions will be discussed in

207 some detail while providing an overview of a large body of work that appeared in the past decade.
208 Finally, flash photolysis studies that helped understand some of the initial details of the peptide
209 cleavage reaction are also presented.

210 4.1. Amino acid or peptide derivatives:

211 Pyrene chromophore was used as the hydrophobic moiety in the design of most of the
212 photoreagents described here, and this choice has been based on the excellent photophysics of this well
213 understood chromophore.²⁹ Pyrene has a high extinction coefficient in the near UV region ($>40,0000$
214 $M^{-1}cm^{-1}$ at 343 nm) and a long lived singlet excited state (>200 ns). The high energy of the pyrenyl
215 excited state can be harvested for photochemical reactions to cleave the peptide backbone. Covalent
216 conjugation of the carboxyl group of 4(1-pyrene)butyric acid with the amine group of L-phenylalanine
217 was done using the standard carbodiimide chemistry³⁰ resulted in phenylalanine-4(1-pyrene)
218 butyramide (Py-L-Phe) (Scheme 2). This was our first photoreagent tested for successful protein
219 photocleavage.

221 *Scheme 2. Synthesis of photocleaving agent Py-L-Phe by linking the COOH group of 4(1-*
222 *pyrenyl)butyric acid with the amine function of L-phenylalanine (Phe) by the standard carbodiimide*
223 *condensation chemistry. Many other amino acids or short peptides can also be used instead of Phe.*

225 Our initial design included the 6-atom linker and the hydrophilic carboxyl group, as in Py-L-
226 Phe, which has a chiral center derived from L-phenylalanine (Scheme 2). Followed by the initial
227 success with Py-L-Phe (described later), the linker length of the photoreagent was systematically tuned
228 by inserting additional amino acid residues (Scheme 3). This variation resulted in a predictable
229 increase in the distance of separation between the pyrenyl chromophore and the hydrophilic carboxyl
230 group. In addition, we also tested logical variations on the amino acid studies of the linker to

231 systematically test the role of the linker in the binding and photocleavage mechanism. Scheme 3
 232 provides a quick overview of the Py-Phe analogs that have been examined in this report.

233
 234 *Scheme 3: Structures of several Pyrenyl photoreagents designed, prepared and examined for protein*
 235 *photocleavage. The corresponding binding constants (K_b) of the probes with BSA are shown in blue.*

236
 237 In addition to changing the linker length, we also examined the subtle influence of the polarity
 238 of the peptide linker (Scheme 3). Conjugation of the carboxyl terminus of the amino acid to the
 239 pyrenyl chromophore, instead, resulted in the corresponding cationic probes, where the polarity of the
 240 amide bond has been reversed. This strategy provided a different perspective of the above method
 241 where the probe might bind to a site that is near, or at, a negatively charged site on the protein instead
 242 of a positively charged site. For example, coupling of the amine group of 4-(1-pyrene) methylamine
 243 (PMA) to the carboxyl group of D/L-Phe resulted in the corresponding PMA-D/L-Phe (Scheme 3). In
 244 another study, the short peptide or the amino acid linker was replaced with biotin to examine if the

245 high affinity biotin group would direct the pyrenyl reagent to its corresponding known binding site on
246 avidin.

247 Guided by these interesting strategies, a small library of photoreagents was synthesized, and
248 their interactions with a small set of proteins have been examined here. The pyrenyl chromophore built
249 into these photoreagents also provided a convenient spectroscopic handle to examine the binding
250 interactions of these reagents with the above proteins. These detailed protein binding studies are
251 described next, prior to the delineation of their protein photocleavage properties.

252 **4.1.1 Photophysical Properties**

253 **4.1.1.1 Absorbance Titrations**

254 The solvent environment surrounding the pyrenyl chromophore has a strong impact on its
255 electronic levels and this attribute is exploited to evaluate the interactions of the pyrenyl probes with
256 proteins.⁷ When a solution of BSA (0-2.0 μ M, 50 mM Tris-HCl buffer, pH 7.0) was titrated into a
257 solution of Py-L-Phe (3 μ M, Tris buffer), a small red shift of 4 nm has been noted.¹ There was a
258 significant hypochromism at 343 nm, and an isosbestic point at 345 nm where the absorbance is
259 independent of the protein concentration (Figure 2).

Figure 2. (A) Absorbance spectra of Py-Phe ($3 \mu\text{M}$) with the addition of BSA ($0\text{--}3.2 \mu\text{M}$), (B) absorbance spectra of Py-Phe ($2.5 \mu\text{M}$) with the addition of lysozyme ($0\text{--}10 \mu\text{M}$), (C) energy level diagram showing the influence of the protein environment on the electronic states, (D) Scatchard plot of Py-Phe with BSA, and (E) Scatchard plot of Py-Phe with lysozyme. Reproduced with permission from *Angewandte Chemie*,³¹ the *Journal of American Chemical Society*,³² and *PNAS*.³³

Because the ground state binds spontaneously to the protein, its free energy is lowered by an amount equivalent to the binding free energy (Figure 2C). As shown by the small red shift in the absorption spectra shows that the energy gap between the excited and the ground state is decreased. One can infer that the excited state has a lower energy in the protein-bound state than in the solvent. Because the gap between the two states is reduced, as indicated by the red shift in the absorption spectra, one concludes that the excited state of the probe binds even better than the corresponding ground state.

The absorption spectral changes of the pyrenyl probes on binding to BSA are summarized in Table 1. All pyrenyl probes that featured a negatively charged end group consistently showed a red shift but there has been no specific trend with the nature of the amino acids in the linker. In general, most of the negatively charged pyrenyl probes seem to interact with a hydrophobic site on BSA in a similar manner, where there has been a bathochromic shift followed by hypochromism. On the other hand, positively charged pyrenyl probes (PMA-D-Phe and PMA-L-Phe) were obtained by the conjugation of 1(1-pyrene)-methyl amine with the carboxyl group of phenylalanine or by conjugation of 4(1-pyrenyl)butyric acid to the carboxyl group of phenylalanine (Phe-Py). These indicated bathochromic shifts but no change in extinction coefficients upon binding to BSA.³⁴ Thus, the terminal group of the linker played a very important role in anchoring the protein at distinct locations but the basis for any preference has not been understood.

286

287 Table 1. Spectral properties of each analog with BSA and lysozyme. Absorption/emission maximum
 288 wavelength is in nm, hyperchromicity/ hypochromicity in percent are given. N/A indicates that the
 289 value is unknown.

Molecule	Absorption/ Emission Max (with BSA)	Hyperchromism/ Hypochromism (with BSA)	Absorption/ Emission Max (with Lysozyme)	Hyperchromism/ Hypochromism (with Lysozyme)
Py-L-Phe	343/378	Hypochromic, 17%	347/378	Hypochromic, 26%
Py-D-Phe	343/378	Hyperchromic	344/378	Hypochromic, 14%
PMA-L-Phe	342/377	N/A	344/378	N/A
PMA-D-Phe	342/377	No Clear change	345/378	Hypochromic
Phe-Py	344/377	N/A	N/A	N/A
Py-Gly	345/377	Hypochromic	345/377	Hypochromic
Py-Gly-His	343/378	Hypochromic, 3%	344/N/A	N/A
Py-Gly-Trp	343/378	Hyperchromic, 33%	346/N/A	N/A
Py-Gly-Tyr	343/378	Hypochromic, 10%	345/N/A	N/A
Py-Gly-Phe	345/377	Hypochromic, 10%	345/377	N/A
Py-Gly-Gly-Phe	345/377	Hypochromic	345/377	Hypochromic
Py-Phe-Gly-Gly	345/377	Hypochromic	345/377	Hypochromic

290

291 In order to examine whether the nature of the protein affects the changes in absorption, the
 292 interaction of the pyrenyl probes with lysozyme has been examined.³ Titration of lysozyme (0-10 μ M)
 293 into a solution of Py-L-Phe (0-50 μ M, Tris buffer) was shown in Figure 2B. In contrast to BSA
 294 (hypochromism, 17% at 343 nm), no red shift occurred in the absorption spectra of Py-L-Phe in the
 295 presence of lysozyme, although considerable hypochromism (26% at 343 nm) was observed.³⁵ When
 296 Py-D-Phe was bound to BSA, hyperchromism (14% at 343 nm) was observed, and there has been
 297 considerable influence of the chiral center on the bound probe properties. Titrations of pyrene
 298 derivatives of increasing linker lengths indicated no spectral shifts, but hyperchromicity has been

noted. Derivatives in which the linker was systematically changed showed no shift in the spectra when bound to lysozyme but they exhibited intense hypochromism (Table 1).

Binding of the cationic derivatives (PMA-L-Phe/PMA-D-Phe) to lysozyme also showed only minor changes in their absorbance spectra. The small hypochromicity indicated that the pyrenyl chromophore resides at a hydrophilic site on the protein, probably exposed considerably to the solvent.² Furthermore, the similarities between the spectral properties of PMA-L-Phe and PMA-D-Phe suggested that both the molecules might reside within the same site on lysozyme.

4.1.1.2 Binding studies

The absorption titration data are useful to determine the intrinsic binding constants of the probes by using a single occupancy, non-interacting, independent, binding site model using Scatchard equation (Equation 1):³⁶

$$(r/C = K(n-r)) \text{ ----- (1)}$$

Here, r is the moles of ligand bound per mole of protein, n is the number of binding sites per protein, and C is the concentration of the free ligand. Linear fits to the data were obtained in all cases examined here, with two typical plots shown in Figure 2D, E. The corresponding K_b and n values extracted from the best fits of the data are shown in Table 2.

Table. 2. Binding constants (K_b) for some pyrenyl probes with BSA.

Py-L-Phe	Py-D-Phe	PMA-D-Phe	PMA-L-Phe	Phe-Py	Py-Gly
$6.5 \times 10^7/\text{M}$	$5.3 \times 10^5/\text{M}$	$4.0 \times 10^6/\text{M}$	$3.8 \times 10^5/\text{M}$	$2.3 \times 10^5/\text{M}$	$1.7 \times 10^6/\text{M}$
Py-Gly-His	Py-Gly-Trp	Py-Gly-Tyr	Py-Gly-Phe	Py-Gly-Gly-Phe	Py-Phe-Gly-Gly
$3.6 \times 10^5/\text{M}$	$6.7 \times 10^5/\text{M}$	$4.3 \times 10^6/\text{M}$	$1.5 \times 10^6/\text{M}$	$1.5 \times 10^6/\text{M}$	$1.5 \times 10^7/\text{M}$

Some interesting comparisons can be made: most ligands had a single binding site with a single occupancy and binding was non-cooperative in all cases examined. Comparisons of the K_b values of these closely related photoreagents provided important insight into the contributions of specific groups of the ligand toward the binding free energy. For example, the K_b value of Py-L-Phe for BSA was two orders of magnitude greater than that of Py-D-Phe, which indicates the strong influence of the chiral center of phenylalanine on the binding interaction. This was surprising, because binding was thought to

323 be dominated by the large hydrophobic pyrenyl group and not by the smaller Phe. These are the first
324 examples of the highest chiral discrimination noted for man-made compounds with any protein.

325 Binding of PMA-L-Phe and PMA-D-Phe isomers with BSA indicated chiral selectivity that is
326 10-fold weaker than that of Py-L-Phe and Py-D-Phe. This decrease may be due to the reversed charge
327 and reversed direction of the peptide linker of PMA-D-Phe and PMA-L-Phe and subsequent binding to
328 a completely different site on BSA. Phe-Py was obtained by conjugating L-Phe with 4(3-pyrene)-
329 butylamine and had the amide bond reversed, and positively charged isomer of Py-Phe. In solutions
330 containing the probe and lysozyme, the selectivity is significantly increased as compared to the
331 selectivity noted for Py-Phe and PMA-D-Phe is favored over PMA-L-Phe. This switch further
332 supports that the chiral center plays an important role in binding site recognition in the protein matrix.²

333 Best fits of the single-site binding model to the binding isotherms indicated that there were two
334 different binding sites for Py-L-Phe on BSA, while there was only one site observed for lysozyme.²
335 The binding capability of each molecule, however, is also affected by the linker length, charge, or
336 hydrophobic element. Scheme 3 as well as Table 2 illustrate that shortening the linker from a 5-carbon
337 chain of Py-Phe to 1-carbon chain of PMA-Phe leads to decreased binding. This decrease was
338 possibly due to insufficient separation of the hydrophobic and hydrophilic groups of the probe across
339 the protein/water boundary. One possibility may be that the hydrophobic pyrene group is substantially
340 exposed to the solvent to the protein surface when the chain is shorter, rather than being buried in the
341 hydrophobic interior of the protein.

342 Binding affinities to BSA were $1.7 \times 10^6 \text{ M}^{-1}$ for Py-Gly, $1.5 \times 10^6 \text{ M}^{-1}$ for Py-Gly-Phe and
343 $1.5 \times 10^6 \text{ M}^{-1}$ for Py-Gly-Gly-Phe. Extension of the hydrophobic segment in the linker from the pyrene
344 chromophore by the addition of Phe, thus contributes to the affinity. This trend, however, was not
345 followed with the Py-Phe or PMA-Phe chiral isomers (Table 2). Py-(Gly)_n-Phe (n = 1 and 2) affinity for
346 BSA decreased 40-fold when compared to that of Py-L-Phe but only 4-fold less for Py-Phe-Gly-Gly.
347 The addition of 1 or 2 glycine residues in Py-(Gly)_n-Phe, gave the same binding affinity as Py-Gly,
348 which is interesting because the additional Phe group did not enhance the affinity significantly.
349 Adding glycine residues to the end of Py-Phe only decreases the binding affinity by 4-fold.⁷

350 Affinities of Py-Gly-X (X=His, Trp, Tyr, Phe) are inversely proportional to the hydrophilicity
351 of the side chain, and the affinities dropped from $4.3 \times 10^6 \text{ M}^{-1}$ to $3.6 \times 10^5 \text{ M}^{-1}$ as the X changed. Where

His < Trp < Phe < Tyr.³⁷ The binding is decreased when a larger hydrophilic element is introduced into the side chain (Tyr, Trp, His) instead of hydrophobic residue, Phe.³⁷ For example, K_b of Py-Gly-His (the most hydrophilic) is an order of magnitude smaller (3.6×10^5) than Py-Gly-Phe (1.5×10^6).³⁷

4.1.1.3 Fluorescence Titrations

The decrease in the energy gap between the ground and first excited state of Py-Phe, noted from the absorption spectra discussed above, suggested a corresponding red shift in the fluorescence spectrum of the pyrenyl probe. Excimer formation, a process where a complex between the ground and the excited states of the same molecule is formed,³⁷ could be examined through the long-lived excited state of the pyrenyl chromophore.²⁹ If the bound probe is isolated from the other probe molecules bound to the same protein or different protein molecules during the excited state lifetime, the formation of the excimer is unlikely. On the other hand, if the probe is rapidly exchanging between the bound and free states, then excimer formation is more likely. Also, if the occupancy at the binding site is 2 or more, then excimer formation is more likely than if the occupancy is 1. Thus, fluorescence studies provided an important avenue to examine binding of the photoreagents to the proteins.

Figure 3. Fluorescence titrations of (A) Py-L-Phe (0-2 μM) and (B) Py-D-Phe (0-2 μM) with BSA (0-8 μM) in 50 mM Tris-HCl Buffer (pH 7.0), upon excitation at 345 nm. Insets show normalized intensity (I_0/I) vs BSA concentration. Reproduced with permission from the Journal of American Chemical Society³² and PNAS.³⁸

372 The fluorescence titrations of the Py-L-Phe (2 μ M) with BSA (0-8 μ M) in 50 mM Tris-HCl
373 buffer, indicated a decrease in fluorescence intensity with increased BSA protein concentration (Figure
374 3).² In contrast, the fluorescence of PMA-L-Phe, PMA-D-Phe, and Phe-Py bound to BSA was
375 enhanced upon binding, which further supported the absorption titration data and suggested
376 considerably different microenvironments for these two different probes in BSA.² Py-Gly-Tyr's
377 fluorescence was enhanced on binding to BSA, but, Py-Gly-His's fluorescence was quenched which
378 suggested binding of these probes to different sites on the same protein and the terminal residue had a
379 significant effect.³⁷ Furthermore, Table 4 shows that fluorescence is significantly lost
380 when the placement of the recognition element is changed, that is, directly attaching it to the
381 chromophore versus including a long linker.

382 In contrast to BSA, binding of Py-L-Phe (2 μ M) to lysozyme (0-10 μ M, 50 mM Tris-HCl
383 buffer, pH 7.0) induced weak quenching of the pyrenyl chromophore at 345 nm, inferring that the
384 binding site lies on the surface of the protein. No peak shifts were observed PMA-L-Phe and PMA-D-
385 Phe was bound to lysozyme.² The fluorescence of probes with different linkers indicated no substantial
386 changes, which is indicative that the binding site is located on the surface of lysozyme, fairly exposed
387 to the solvent in all cases studied here. Titrations of the Py-(Gly)_n-Phe analogues of increasing chain
388 lengths also indicated weak quenching of their fluorescence with no shifts in their spectral maxima.⁷

389 In all of the above cases, we did not find excimer emission in the presence of lysozyme, which
390 suggested that the pyrenyl chromophore is buried in the protein in its monomeric form (single
391 occupancy).⁴ However, with BSA, excimer formation was observed in some cases (Py-L-Phe, Phe-Py
392 and Py-Gly-Gly-Phe), which indicated that the pyrenyl chromophore is buried in the protein in its
393 dimeric form or two of the probes could find each other within the excited state lifetime.^{7,35,37}

394 **4.1.1.4 Circular Dichroism Studies**

395 Many of the pyrenyl probes investigated here had intrinsic CD bands in the region of the
396 pyrene absorption spectra from one or more asymmetric centers in their side chains (Figure 4, Table 3).
397 Close interaction of the probes pyrenyl chromophore with the chiral environment of the protein was
398 expected to have a strong influence on the intrinsic CDs of the pyrenyl probes. The isomers Py-L-Phe
399 and Py-D-Phe, for example, exhibited CD spectra that were near-mirror images of each other in the
400 absence of the protein (Data not shown).³⁵ Upon binding to BSA (55 μ M) the CD peaks of both

401 isomers were inverted with peak positions at 327 nm and 345 nm for the L isomer and at 330 nm and
402 347 nm for the D isomer (Figure 4A).⁴

403 The CD intensity when bound to BSA decreased for PMA-L-Phe and increased for PMA-D-Phe
404 (Figure 4C, D).² Py-Gly had no asymmetric center and thus had no intrinsic CD bands but when bound
405 to BSA it indicated induced CD bands at 314, 334 and, 350 nm. Therefore, the induced CD confirms
406 the asymmetric environment around the pyrene chromophore in the bound state even when it is not
407 intrinsic to the probe.

408 Although the absorbance and fluorescence spectra of Py-(Gly)_n-Phe bound to BSA are similar,
409 their CD spectra varied substantially, providing a unique “fingerprint” for each probe. The variation
410 comes from changes to their microenvironments at the binding site such as, the orientation of the
411 pyrenyl chromophore with respect to its own asymmetric center, or the protein asymmetric
412 environment around it.⁷ When the linker length is increased, as in the case of Py-Phe-Gly-Gly, the CD
413 ellipticities changed only marginally (Figure 4E). Lastly, the CD spectra reported here are unique to a
414 given protein and the probe. With respect to lysozyme, only minor changes were observed (Figure 4B).

Figure 4. CD spectra of the Py-Phe family of probes bound to the target proteins. In all cases concentrations were 50 μ M Py-Phe, 50 μ M protein in Tris buffer (50 mM, pH 7.2), unless otherwise stated. (A) Py-Phe bound to BSA, (B) Py-L-Phe bound to lysozyme, (C) free PMA-D-Phe and PMA-L-Phe (30 μ M) in Tris Buffer, (C) PMA-D-Phe and PMA-L-Phe (30 μ M) bound to BSA or lysozyme, (D) free PMA-L-Phe (30 μ M, solid line) and bound BSA or lysozyme (bold and dashed lines, respectively), (E) Py-Phe-Gly-Gly in the absence (dotted line) and presence of BSA (solid line), (F) Py-Gly bound to BSA or lysozyme, and (G) free Py-Gly-Tyrosine (dotted line), Py-Gly-Tyrosine bound to BSA (dashed line) and free BSA (solid line).

One overarching observation could be made from the small set of CD spectra shown in Figure 4 and Table 3. No two probes showed the same CD spectrum with BSA and no two proteins showed

the same CD spectrum a given probe. This outcome is mostly likely due to the unique chiral environment the probe experiences while residing in the protein matrix. These spectra could serve as ‘fingerprints’ for a given protein or for a given probe. But this inference needs to be tested further for any practical applications of CD spectra for the identification of the probes or the proteins.

Table 3. Circular dichroism bands (nm) of Py-Phe and its derivatives

Molecule	Negative CD Peaks (nm)	Positive CD Peaks (nm)
Py-D-Phe	330 and 347 (bound to BSA)	--
Py-L-Phe	336, 351, and 360 nm (Free Probe)	327 and 345 (bound to BSA)
PMA-D-Phe	--	330 and 346 (bound to BSA) 328, 344 and, 354 (bound to Lysozyme)
PMA-L-Phe	328 and 344 (bound to BSA) 328 and 342 (bound to lysozyme)	--
Py-Gly	314, 334 and, 350 (bound to BSA)	--
Py-Phe-Gly-Gly	341 and 357 (Free probe) 342 and 358 (bound to BSA)	--

4.1.1.5 Fluorescence quenching Studies

Solvent accessibility of the protein-bound photoreagent is crucial in our strategy of achieving photocleavage reaction. That is, the pyrenyl chromophore is to sensitize the photodecomposition of CoHA to generate reactive amine radical which could abstract a hydrogen atom from the peptide backbone and initiate the cleavage chemistry. Therefore, quenching of fluorescence from the protein-bound pyrenyl probes by CoHA were assessed, systematically. The corresponding Stern-Volmer quenching constants (K_{SV}) have been determined for the photoreagents by fluorescence quenching studies and compared (Table 4). For example, the quenching constants were calculated using the

442 fluorescence intensities in the presence and absence of CoHA using the Stern-Volmer equation
443 (equation 2),

444
$$I_0/I = 1 + K_{sv}V[Q] \text{ ----- (2)}$$

445 Where [Q] is the quencher concentration, and K_{sv} is the Stern-Volmer constant, I is the fluorescence
446 intensity in the presence of specific concentration of CoHA and I_0 is the corresponding value in the
447 absence of CoHA.³⁹ Linear relationships were observed in almost all cases when plotting I_0/I vs
448 CoHA concentration, using the Stern-Volmer equation. K_{sv} decreased when the photoreagent was
449 bound to the protein, depending on the degree of accessibility of the probe to the aqueous media,
450 excited state lifetime as well as the diffusion coefficient of the quencher.

451 *Table 4. Fluorescence quenching by CoHA for probes bound to BSA or lysozyme, as indicated (M^{-1}).*

Probe	Py-L-Phe	Py-D-Phe	PMA-L-Phe	PMA-D-Phe	Py-Gly	Py-Gly-Phe	Py-Gly-Gly-Phe	Py-Phe-Gly-Gly
K_{sv}/M (free Probe)	1.9×10^3	1.9×10^3	1.28×10^3	2.31×10^3	1.3×10^3	1.3×10^3	1.9×10^3	1.3×10^3
K_{sv}/M (2 μM BSA)	0.25×10^3	Intensity enhanced	Intensity enhanced	0.15×10^3	0.31×10^3	0.38×10^3	<10	<10
K_{sv}/M (2 μM lysozyme)	1.4×10^3	0.53×10^3	0.79×10^3	0.72×10^3	1.2×10^3	1.0×10^3	0.36×10^3	0.82×10^3

452

453 The quenching data collected in Table 4 clearly shows that Py-L-Phe and Py-D-Phe, for
454 example, are quenched with equal efficiency in the absence of the protein. On binding to the protein,
455 however, the efficiency changes dramatically; Py-L-Phe when bound to BSA or lysozyme quenched
456 the fluorescence by 13% or 74% respectively. The decrease in the K_{sv} which occurs when the probe
457 binds to the protein, points to the extensive decrease in accessibility of the probe to the quencher, when
458 the quencher is present in the solvent.³ We assume that CoHA does not bind to the protein, and this
459 assumption may not be valid in some specific cases when the quencher also binds to the protein. Large
460 decrease in the quenching constant implies extensive protection of the probe from the quencher by the

461 protein, and it is less likely that the excited probe would sensitize the photodecomposition of CoHA.
462 The photocleavage yield, therefore, expected to be low.

463

464 *Scheme 4: Differences in the solvent exposure of photoreagents when bound to lysozyme vs BSA. The*
465 *black rectangle represents the chromophore, the blue ball represents the polar head group, and the*
466 *black squiggle represents the linker.*

467

468 With Py-L-Phe (2 μM) bound to BSA (2 μM), the emission is weakly quenched ($K_{\text{sv}} = 0.25 \times 10^3$),
469 unlike its counterpart in which the emission is enhanced (Table 4). The quenching constants for
470 cationic probes such as PMA-Phe observed the opposite trend; with the L isomer having enhanced
471 fluorescence and the D isomer being quenched ($K_{\text{sv}} = 0.15 \times 10^3$). For probes in which the linker length
472 was changed, the efficiency changes as the distance between the recognition element and the
473 chromophore are adjusted. The fluorescence quenching constants for the BSA-bound probe decreased
474 with increasing linker length.⁷ In the presence of lysozyme (2 μM), Py-L-Phe (2 μM) is quenched
475 more efficiently than the D isomer. PMA-D-Phe and PMA-L-Phe showed little difference in
476 quenching constants when bound to lysozyme ($K_{\text{sv}} = 0.72 \times 10^3$ and 0.79×10^3 respectively).² The K_{sv}
477 values for analogues containing different lengths of linker were significantly larger than the ones
478 observed in the presence of BSA. K_{sv} values (Table 4) indicate that the probe binds near surface of
479 lysozyme with good exposure to the solvent. This continues to show the ease of accessibility of the
480 probe to solvent-bound CoHA at least when the probe was bound to lysozyme. Based on these studies,
481 we proposed that probes bound to BSA are, in general, well-protected by the protein while the probes
482 bound to lysozyme are much more accessible to the solvent-bound CoHA (Scheme 4).^{3,35} These
483 deductions are important and explain the greater yields of protein cleavage products with lysozyme
484 when compared to those with BSA.

485 **4.1.1.6. Chiral discrimination and fluorescence lifetime studies**

486 An unexpected outcome of our studies has been achieving high chiral discrimination of
 487 photoreagent binding to the proteins, for a man-made molecule. Py-L-Phe, for example, indicated a
 488 binding affinity for BSA which is nearly 100 times greater than the corresponding D-isomer, Py-D-Phe
 489 (Table 2).⁴⁰ The chiral discrimination is also evident from the absorbance and fluorescence spectral
 490 titrations, discussed earlier. The absorbance spectrum for Py-L-Phe, for example, showed
 491 hypochromism when bound to BSA, while Py-D-Phe showed hyperchromism.^{4,6,35} Fluorescence
 492 spectra also showed a similar contrast in that Py-L-Phe intensity decreased on binding to BSA while
 493 that of Py-D-Phe has been enhanced. Furthermore, time resolved fluorescence data showed that when
 494 bound to BSA, the lifetimes of these two probes are different (Figure 5). Their lifetimes in solution
 495 were identical (105 ns, air saturated solutions) and hence, the differences arose upon their binding to
 496 the protein. Furthermore, the fluorescence lifetimes increased on binding to the protein. Figure 5
 497 shows two distinct components of ~70 and 153 ns for the L isomer and ~70 and 168 ns for the D
 498 isomer.⁴⁰ The two isomers did not differ in terms of their photoproduct yields, described later. Chiral
 499 discrimination of Py-Phe isomers noted with lysozyme, however, has been substantially greater than
 500 that noted with BSA.⁴⁰

501
 502 *Figure 5. Fluorescence decay curves of Py-L-Phe and Py-D-Phe (2 μM) in the absence and presence of*
 503 *BSA (2 μM). Py-L-Phe and Py-D-Phe bound to BSA showed biexponential decays. Reproduced with*
 504 *permission from PNAS.*³⁸

505

506 **4.1.2 Photochemical Studies**

507 Our major goal for these studies was to evaluate the photochemical cleavage of proteins by
508 these photoreagents and quantify the details of the photoreaction. Knowing the identity of the
509 photoreagent binding site on the proteins is an important detail to fully understand the above
510 experimental data and the photocleavage mechanism. These details were assessed directly from
511 photochemical studies by irradiating a mixture of the photoreagent, the quencher and, the protein at
512 appropriate wavelengths for substantial duration. The pyrenyl chromophore by itself is not photoactive
513 and Py-Phe does not cleave proteins when irradiated with near UV light (~340 nm). The long lived
514 excited state of the probe and its facile access to CoHA suggested that sensitized photochemical
515 reaction could be used to induce protein photocleavage. Since CoHA has been known to undergo
516 photodecomposition giving rise to NH_2 radicals,⁴ we imagined that photogeneration of these reactive
517 species at the probe binding site could initiate peptide bond cleavage. The photocleavage of the protein
518 backbone is then expected to form smaller fragments than the parent, which could be separated by
519 standard protein gel electrophoresis (SDS/PAGE) studies. The newly formed amino terminus of the
520 peptide was amenable to amino acid sequencing, therefore, giving the exact details of the cleavage
521 patterns of the photoreactions. This unexpected feature is useful in biochemical applications of these
522 photoreagents.

523 **4.1.2.1 SDS/PAGE Studies**

524 Solutions containing CoHA (1 mM), BSA (15 μM) and Py-L-Phe (15 μM) (50 mM Tris-HCl buffer,
525 pH 7.0, total volume 200 μL) were irradiated at 343 nm for increasing periods of time, using a 150 W
526 xenon lamp with a cutoff filter (WG-345) for removing of stray UV light. Py-Phe/BSA had an
527 absorbance maximum at this wavelength and the photoreaction progress has been monitored by
528 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) studies. The products are
529 separated by applying electric field and their migration through the gel depends on their size.⁴¹ The
530 reaction mixtures were loaded onto the gel, electrophoresed, and after developing the gel bands by
531 appropriate stain, there appeared sharp protein bands. In most cases we examined, these gels indicated
532 the formation of just two product bands of lower molecular weight than the parent protein (Figure 6A,
533 lanes 4-7), in addition to the band of the unreacted protein. Control lanes with no CoHA (Figure 6A,
534 Lane 9) or no Py-Phe (Figure 6A, Lane 10) or no light (Figure 6, Lane 3) did not indicate any product.
535 Thus, the photoreaction required light, CoHA, the photoreagent and the protein and resulted in a pair
536 of products due to a single cut in the peptide backbone. The sum of the molecular weights of the two

product bands equaled to that of BSA, further establishing that there has been a single cleavage site. If the photocleavage was random, then these bands would have been a smear and the sum of the product molecular weights would differ from that of BSA.¹ The photochemical product yield was calculated through quantification of the gel using NIHimage software (v 1.6), in which the total intensity of the fragment bands were divided by the total intensity in the entire lane.

Figure 6. A. SDS/PAGE gel of BSA photoreaction. Lane 1: Molecular weight markers in kDa, Lane 2: BSA (15 μM), Lanes 3-7: BSA+Py-L-Phe+CoHA, all irradiated for 0 (dark control), 10, 20, 30 and 60 minutes at 344 nm, Lane 8: BSA, Lane 9 BSA+Py-L-Phe, Lane 10: BSA + CoHA, Lanes 8-10 irradiated for 30 minutes. B. SDS/PAGE gel of lysozyme photoreaction. Lane 1: Molecular weight markers in kDa, Lane 2: Lysozyme (15 μM), Lanes 3-6: Lysozyme+Py-D-Phe+CoHA irradiated for 0,5,10, and 20 minutes at 344 nm, Lane 7: Lysozyme, Lane 8: Lysozyme+ Py-D-Phe, Lane 9: Lysozyme+CoHA, Lanes 7-9 irradiated for 20 minutes. Reproduced with permission from *Angewandte Chemie*³¹ and *PNAS*³⁸.

To test if the photoreaction is just unique to BSA or more generally applicable, we also examined the photoreaction of lysozyme with Py-D-Phe. Since this probe binds to lysozyme and its excited state has been accessible to CoHA, shown in quenching studies, this was a reasonable expectation. Irradiation of Py-D-Phe/lysozyme mixture (50 mM Tris-HCl buffer, pH 7.0) at 334 nm resulted in two distinct products as noted in the SDS/PAGE (Figure 6B). These are of lower molecular weight than that of lysozyme. Lanes containing dark control (lane 3) showed no reaction as the lanes that contained just protein and Py-D-Phe or protein and CoHA but irradiated with light (lanes 8 and 9). Irradiation time is optimal at 20 minutes, after that photocleavage reached a plateau. Furthermore, the sum of the molecular weights of the two bands equaled that of the parent protein, indicating that a single cut has been made (Figure 6B, lanes 4-6).³⁵ Not only does Py-D-Phe photocleave lysozyme at a single site, but it also had among the highest photoproduct yields (21%, Table 5). This increased yield is consistent with higher Stern-Volmer quenching constants noted with lysozyme when compared to

those of BSA. From these gels of BSA and Lysozyme, we conclude that the reaction requires all three components, light, CoHA, and Py-Phe to proceed.

Table 5. The photocleavage yields for BSA and lysozyme with the Py-Phe and its derivatives.

Probe/Protein	BSA	Lysozyme
Py-L-Phe	25%	35%
Py-D-Phe	25%	21%
Phe-Py	<5%	42%
PMA-L-Phe	<5%	57%
PMA-D-Phe	<5%	52%
Py-Gly-Phe	<5%	21%
Py-Gly-His	5%	15%
Py-Gly-Tyr	<5%	7%
Py-Gly-Trp	<5%	<5%
Py-Phe-Gly-Gly	5%	22%
Py-(Gly) _n -Phe (n=1,2)	5%	17%

Along these lines, we examined the photoreactivities of Py-D-Phe, PMA-L-Phe and PMA-D-Phe, with both BSA and lysozyme and all of which produced a single pair of photoproducts in good to moderate yields (Table 5). The latter indicated much weaker product yields but there has been no chiral discrimination in terms of the product yields. However, the product yields for the photocleavage reaction differ significantly (Table 5). Photoproduct yields of BSA with Py-Phe-Gly-Gly (yields <5%, irradiated at 344 nm for 60 minutes) are lower than those observed with lysozyme (22%), because the probe bound to lysozyme is exposed to the solvent and its emission has been more efficiently quenched by CoHA. In contrast, the probes bound to BSA are buried in a deep hydrophobic cavity, making them less accessible to CoHA, as indicated by the fluorescence quenching data. Even in the case of BSA, where the probes are less accessible, there has been substantial photocleavage resulting in a single pair of product bands. Yields for lysozyme/PMA-Phe photoreactions (Figure 7A) were quite high (57%) even when compared to that of lysozyme/Py-L-Phe (35%)² (Table 5), which are consistent with the fluorescence quenching studies discussed earlier. Photoreaction yields were also calculated for the cleavage of lysozyme by Phe-Py (Figure 7B), a molecule similar to PMA-Phe in that the charge is

reversed (Table 5). Interestingly enough, Phe-Py cleaved lysozyme in a very similar fashion to PMA-Phe (Figure 7 A, B)

Next, we examined the role of the peptide side chain in controlling the photoproduct formation with Py-Gly-Phe, Py-Gly-Tyr, and Py-Gly-His (Table 6).⁶ Py-Phe and Py-Gly-His showed considerable yields, while the Tyr and Trp analogs did produce any photoproducts. Flash photolysis studies showed that the Tyr and His residues quench the initially produced radical intermediate in the mechanism (discussed later), thus quenching the reaction at the initial stages. The photoproduct yields of Py-Gly-X derivatives (where X was one or more amino acids shown in Table 6) with lysozyme were higher than the corresponding values noted with BSA (Table 6). Increased photoproduct yields are consistent with increased access of the chromophore to CoHA.

Figure 7. (A) SDS/PAGE of lysozyme photocleavage with PMA-Phe upon excitation at 342 nm. Lane 1: Protein Marker, Lane 2-5: 15 μM lysozyme, 15 μM PMA-Phe, 1 mM CoHA irradiated for 0, 10, 20 minutes, Lane 6: 15 μM lysozyme and 15 μM PMA-Phe irradiated for 30 minutes. (B) SDS/PAGE of lysozyme cleavage with Phe-Py, upon excitation at 344 nm. Lane 1: Protein Marker, Lane 2-5: 5 μM lysozyme, 2 μM Phe-Py and, 0.3 mM CoHA irradiated for 0, 10, 20, or 30 minutes, Lane 6: 5 μM lysozyme and 2 μM Phe-Py irradiated for 30 minutes (C) SDS/PAGE gel of BSA cleavage with Py-(Gly)_n-Phe analogues upon excitation at 344 nm. Lane 1: protein marker, lane 2: BSA, Py-L-Phe, CoHA (Dark control), Lane 3: BSA, Py-L-Phe, CoHA Lane 4: BSA, Py-Gly-Phe, CoHA, Lane 5: BSA Py-Gly-Gly-Phe, CoHA, Lane 6: BSA, Py-Phe-Gly-Gly, CoHA. Samples in lanes 3-6 were irradiated for 60 minutes. Reproduced with permission from the Journal of American Chemical Society.³²

Next, we examined the influence of the linker length on the photocleavage yield because a greater distance between the hydrophobic and hydrophilic groups in the probe should bury the pyrenyl group further into the protein interior. This should bury the pyrenyl chromophore further into the protein matrix and cleave the protein further down the peptide chain. This simple strategy could allow

us to tune the location of the cleavage site. Py-(Gly)_n-Phe analogues (n= 0, 1, 2) are used to test this strategy. The yields of the photoproducts with BSA as the target protein decreased for n=0, 1 and 2, (Figure 7C, lanes 4-6) when compared to that of Py-L-Phe.⁷ These observations were, again, consistent with the quenching data presented earlier.

612

613 **4.1.2.1 Quantum yield measurements**

614 The quantum yield of the photocleavage reaction of Py-L-Phe with lysozyme was 0.26, which
615 is quite respectable considering that it is a photoreaction with a macromolecule.^{3,6} The quantum yield
616 of photocleavage of BSA by Py-L-Phe was 0.0021, which is much less efficient than that of lysozyme
617 for reasons discussed above.^{3,6}

618

619 **4.1.2.2 Peptide Sequencing Studies**

620 Sequencing the newly produced product peptides would reveal the exact location of the
621 cleavage site on the protein, and identifying the photocleavage site is an important step in
622 understanding the mechanism as well as for applications in protein photo-sequencing studies.^{2,3,5,7,35}
623 This was done by standard biochemical methods of amino acid sequencing.⁴² Western blotting of the
624 photofragments separated on SDS-polyacrylamide gel were transferred to PVDF membrane by
625 applying a current of 60 mA for 1.5 h by the semi-dry system (BIORAD) in CAPS (3-
626 (cyclohexylamino)-1-propanesulfonic acid) buffer, pH 10.5. The transferred protein bands were
627 stained with Coomassie Brilliant Blue, bands were cut, and submitted for N-terminal sequencing. The
628 newly formed N-terminus of the product band was sequenceable, in all the cases we have examined so
629 far, and this is a good outcome for the identification of the photocleavage sites. In some cases, the
630 newly formed C-terminus was blocked from sequencing but both the original N-terminus and C-
631 terminus were sequenceable. These termini were not damaged during the photoreaction and the
632 sequencing data confirmed the identities of the product bands.

633 Table 6. Photocleavage sites of pyrenyl probes with BSA and lysozyme.

634

Probe/Protein	BSA Cleavage Site	Lysozyme Cleavage Site
Py-L-Phe	Leu346-Arg347	Trp108-Val109
Py-D-Phe	Leu346-Arg347	Trp108-Val109

PMA-L-Phe	Not Sequenced	Trp108-Val109 and a minor site
PMA-D-Phe	Not Sequenced	Trp108-Val109 and a minor site
Py-Gly-Phe	Leu346-Arg347	Trp108-Val109
Py-Gly-His	Leu346-Arg347	Trp108-Val109
Py-Gly-Tyr	Leu346-Arg347	Trp108-Val109
Py-Gly-Trp	Leu346-Arg347	Trp108-Val109
Py-Phe-Gly-Gly (n=1,2)	2 major sites, not sequenced	Trp108-Val109 and Ala110-Trp111

Amino acid sequencing of photo fragments of the BSA/Py-L-Phe photoreaction indicated that the protein is cleaved at Leu-346 and Arg-347 (Table 6), a site that is buried within the protein. To our surprise, both L and the D isomers cleaved at exactly the same site, despite the differences in their binding properties noted earlier. There were no other detectable products in the sequencing analysis, thus corroborating with the above observation that BSA was cleaved at a single site by both Py-L-Phe and Py-D-Phe. That is, the photocleavage has been site specific, an unexpected success, given the great many possible sites the probe could have bound on the protein or cleaved at any of the numerous amide linkages.

Amino acid sequencing of Py-(Gly)_n-Phe and its analogues indicated that that the cleavage site is the same for most of the probes examined here, for a given protein. Only occasionally, there have been minor differences noted. That is, the photocleavage is not amino acid sequence dependent but protein site dependent. The BSA fragments were separated, purified, and sequenced in many cases. Based on SDS-PAGE results of photocleavage reactions with the analogues and BSA (Figure 7), there appears to be a dependence on the probes structure in terms of how efficient the probe is and the location at which it cleaves. For example, Py-Gly-Phe and Py-Gly-Gly-Phe (Figure 7C, lane 4 and 5) showed 3 product bands while Py-Phe-Gly-Gly shows just 2 product bands (Figure 7C, lane 6).⁷ The changes in the photoproducts indicate that the selectivity varies from probe to probe. All probes examined here, cleaved lysozyme between Trp108 and Val109, and in the cases of Py-(Gly)_n-Phe and Py-Phe-Gly-Gly a minor cleavage site was noted at Ala110 and Trp111.

Similarly, N-terminal sequencing of the fragments from Py-L-Phe/lysozyme indicated that the protein was cleaved between Trp108-Val109, a site internal to lysozyme, again there were no other

657 products in the sequencing analysis. Both L and D isomers cleaved at the same site, and both cleaved
658 lysozyme at a single site, site specific (Table 6).³⁷

659 The photocleavage site of PMA-Phe on lysozyme was determined to be at Trp108-Val109 for both L
660 and the D isomers but noted another very minor fragment with the D isomer, which could not be
661 identified due to its low yield.² However, this minor product was not observed with the L-isomer or
662 with the Py-Phe isomers discussed above (Table 6).

663 Amino acid sequencing studies of Py-(Gly)*n*-Phe analogues indicated that the major cleavage
664 site on lysozyme was Trp108-Val109 as with Py-Phe, but it also indicated a minor site between Ala110
665 and Trp111^{35,7}. Increasing the length of the linker resulted in a change of cleavage site, as well as loss
666 of site specificity (Table 6). Thus, the linker length does influence binding affinity to the protein, site
667 of binding and photocleavage properties of the probes.

668 4.1.3 Computational Modeling

669 Next, we examined the binding of specific photoreagents to the target proteins by
670 computational modeling to gain a better understanding of how the protein fragmentation occurred at
671 the specific sites identified in the sequencing studies. The availability of powerful modern software
672 and high capacity computational clusters made this type of modeling routine in our laboratories, and
673 hence some of these unpublished details are presented here. Computational modeling has been, luckily,
674 in good agreement with the established cleavage sites of the probes described here, thus validating the
675 modeling studies. Modeling studies may be used in future studies, with caution, prior to the
676 photochemical studies, and this strategy could provide an iterative method to refine probe design based
677 on the desired target site.

678 The modeling studies used the known crystal structures of the proteins such as BSA (PDB file
679 name, 4OR0), obtained from the Protein Data Bank and any ligands present in the protein has been
680 removed. The photoreagent structure was optimized by Gaussian 03 at HF/3-21G level of calculation⁴³
681 and AutoDock 4.2 has been used to calculate the interaction energy between the probe and the protein,
682 after energy minimization. The size of the docking box was set at 120 x 120 x 120 Å³ with the spacing
683 of 0.375 Å. The center of grid box was located at the center of macromolecule with the coordinate
684 center (x, y and z) of 61.553 26.428 and 90.152, respectively. The docking results were viewed by
685 Accelrys Discovery Studio Client 2.5.

Figure 8. Docking study of BSA with Py-L-Phe (A) and PMA-L-Phe (B). Py-L-Phe indicates hydrogen bonding interactions with Lys350, Leu480, Val481 and hydrophobic interaction with Trp213, while PMA-L-Phe indicates hydrogen bonding interactions with Ala209 and hydrophobic interaction with Trp213.

The docking results with BSA/Py-L-Phe are shown in Figure 8A, which supported the observed photocleavage site of Leu346-Arg347 in domain II. Modeling showed that this site is located within an alpha helical region, and clearly indicated the strong possibility of stacking interactions between the pyrenyl chromophore of Py-Phe and Trp213. The computed binding free energy of -13.5 kcal/mol compared well with the binding studies described earlier. Using the equation 3:

$$\Delta G = - RT \ln K_b \text{ -----(3)}$$

Where $R = 1.9872036 \text{ calK}^{-1}\text{mol}^{-1}$, $T = 273 \text{ K}$, experimental binding energies were calculated (Table 7). Although lower than the ones determined using computational modeling, but they follow the same trend. Hydrogen bonding interactions between the Py-L-Phe and lys350, leu480, val481 are clearly supported, and the cleavage site is in good proximity to the phenyl group of Py-L-Phe (Table 7).

Table 7: Computational calculations of Distance between Pyrene and the cleavage site (Å) and the binding free energy (kcal/mol)

	Distance	Distance	BSA Binding	Lysozyme Binding	Binding Free
--	----------	----------	-------------	------------------	--------------

	between Py to BSA Cleavage site (Å)	between Py to lysozyme Cleavage site (Å)	Free Energy (k cal/mol) (Calculated)	Free Energy (k cal/mol) (calculated)	Energy (kcal/mol) (experimental)
Py-L-Phe	3.719	3.083	-13.25	-8.95	-9.76 (BSA)
Py-D-Phe	3.993	3.510	-12.79	-9.38	-7.15 (BSA)
PMA-L-Phe	--	3.742	-11.11	-11.05	-6.97 (BSA)
PMA-D-Phe	--	3.282	-11.27	-11.09	-8.25 (BSA)

In a similar manner, we identified the docking site of PMA-L-Phe on BSA (Figure 8B), which binds with a lower affinity ($\Delta G = -11.11$ kcal/mol) than Py-L-Phe. The docked site is in good proximity to the observed cleavage site, but this structure indicated hydrogen bonding with Ala209. The differences of the binding free energies between these two probes, at the same site, could result from the large differences in the conformation of the photoreagents and the distances from the residues that interact with them (Table 7). These differences, however, did not change the photoproduct identity but PMA-Phe isomers had a much lower photocleavage yields than Py-Phe isomers, which suggests that the conformation of the bound photoreagent in the binding pocket is important in determining its reactivity.

4.1.4 Flash Photolysis Studies and the Mechanism

Laser flash photolysis was used to study the mechanism of the photoreaction and examine the intermediates formed subsequent to the photoexcitation.^{3,35,37} Light pulses of ~10 ns width at 355 nm were used to excite solutions of 20 μ M Py-L-Phe, 3 mM CoHA and 30 μ M BSA or lysozyme (Figure 9).

Figure 9. Transient absorbance spectra recorded at 1-7 μs delay after excitation at 355 nm of air saturated 20 μM Py-D-Phe or Py-L-Phe solutions with (A) and without (B) 3 mM CoHA. Insets show the kinetic traces at 460 nm (A) or 420 nm (B). Reproduced with permission from PNAS.³⁸

Light excitation was followed by rapid rise of a transient with absorption maxima at 405, 420, 460, 490, and 510 nm for BSA, while the corresponding lysozyme/probe samples had bands at 390, 425, 470, and 510 nm. The same time-resolved spectra were noted for either of the Py-D-Phe isomers, and hence, both reactions generated the same reactive intermediates. This transient has been identified, next, in kinetic studies described below.

Kinetic traces recorded at the absorption maxima of the above transient showed that the band corresponding to 460 nm had a half-life of 50 μs (Figure 9A, inset). This intermediate has been identified as the pyrene cation radical, from a number of quenching studies and control experiments. This transient was also formed when Py-Phe excited state was quenched by 3 mM CoHA, in the absence of the protein.^{3,3737} Thus, this intermediate does not originate from the protein or its side chains, but derived from the probe itself. The 460 nm transient was also not quenched by oxygen to any significant extent, ruling out the possibility that it could be the Py-Phe triplet state. In the absence of CoHA, both Py-L-Phe and Py-D-Phe produced a transient with an absorption maximum at 420 nm and a lifetime of 18 μs (Figure 9 B). The transient at 420 nm was quenched by oxygen at diffusion controlled rates, and has been identified as the pyrene triplet state. The 460 transient was absent under these conditions.

Figure 10: Proposed mechanism for protein photocleavage by Py-Phe and its derivatives.

Thus, our hypothesis of CoHA quenching the probe excited state and producing the corresponding cation radical is supported by the flash photolysis data. The proposed mechanism^{2,3,7,35} (Figure 10) is consistent with the observations that the newly formed N-terminus is sequenceable as well as the cleavage always occurred next to an amino acid bearing beta hydrogens. Many alternative mechanisms could be written for this reaction, but most of these do not account for the presence of free N-terminus of the daughter fragment or justify the need for an accessible β hydrogen at the cleavage site. The pyrene cation radical formed from the probe excited state by quenching with CoHA might lose a proton and become the corresponding aryl radical, which could react with oxygen to form hydroperoxyl radical that could eventually lead to peptide backbone cleavage.^{2,3,7,35} Due to the large size and complex structure of the protein, more detailed studies are required to identify further details of the photocleavage mechanism arising from the cation radical.

4.2. Avidin-based Pyrenyl Probes

Next, we tested if the pyrene probe could be directed to specific sites on the protein by linking it to biological ligands that are known to bind to a particular target protein, at known sites. Biotin, for example, is known to bind to avidin, a tetrameric glycoprotein with a molecular weight of 15 kDa per one subunit.²⁵ The binding constant for this interaction is very high (10^{15} M^{-1}), one of the strongest noncovalent protein-ligand interactions known. Therefore, we decided to conjugate the pyrene

765 chromophore to biotin, and test if the binding is dominated by the hydrophobic pyrene or the high
766 affinity biotin with avidin.

767 4.2.1 Synthesis and Characterization of Py-Biotin

768 Coupling of 1(1-pyrene)methylamine hydrochloride with d-biotin by using *N,N'*-dicyclohexyl
769 carbodiimide⁵ (Scheme 5) produced d-biotinyl-1(1-pyrene) methylamide (Py-Biotin), with
770 characterization data as follows: ¹H NMR (400 MHz; d₆-DMSO): 7.8–8.2 (9H), 5.0 (1H), 4.1 (1H), 3.8
771 (1H), 3.3 (2H), 2.5–2.6 (2H), 2.2 (2H), 1.1–1.7 (6H); MS data: *m/z* 456.4 (M⁺), which are consistent
772 with the assigned structure.⁵ Next, we examined the binding and photoreactions of Py-Biotin with
773 avidin as the corresponding substrate to test if the binding is dominated by the pyrene chromophore or
774 the biotin, which is a popular biological recognition element.

776 Scheme 5. Structure of d-biotinyl-1(1-pyrene)methylamide (Py-Biotin), synthesized by coupling of d-
777 biotin to 1(1-pyrene)methylamine hydrochloride (Amide bond highlighted in red).

778

779 4.2.2 Photophysical studies of Py-Biotin with Avidin

780 4.2.2.1 Absorbance Titrations:

781 Pyrenyl chromophore linked to biotin, in the form of Py-Biotin, is expected to be delivered to
782 the specific site on avidin because the binding affinity of biotin for its site on avidin should far exceed
783 the binding affinity of pyrene. This is because biotin is known to have the strongest affinity for any
784 known ligand for a protein.²⁵ Addition of avidin solution (0–1.5 μM) to a solution of Py-biotin (4.2
785 μM), resulted in a small red shift in the absorbance spectrum and hyperchromism with an absorption
786 maximum at 342 nm.⁵

787 4.2.2.2 Fluorescence Titrations

788 The fluorescence of Py-Biotin is enhanced by the addition of avidin (0-10 μM) to Py-biotin
 789 solution (2 μM , excitation at 342 nm, data not shown), indicating that protein/Py-biotin complex is
 790 formed.⁵ No excimer emission or peak shifts were observed in the spectra. The enhanced emission
 791 could be because the binding site is buried within the protein and protected from the solvent. The
 792 decreased accessibility of Py-Biotin to the solvent was probed by using CoHA as a quencher and the
 793 K_{sv} is dramatically reduced from 1400 M^{-1} (free probe) to 170 M^{-1} (bound probe, Figure 11B)⁵. This
 794 large decrease in access to CoHA could negatively impact photocleavage reaction because this
 795 quenching is essential for the photoreaction.

796 A. B. C.
 797 Figure 11. (A) Fluorescence spectra of Py-biotin (2 μM) with increasing concentrations of CoHA (0-
 798 1.0 mM). Excitation wavelength is 342 nm. (B) Quenching plots of the Py-biotin by CoHA, in buffer
 799 (closed circles), and in the presence of avidin (squares). (C). Photocleavage of avidin by Py-Biotin
 800 upon excitation at 342 nm. Lane 1: Protein marker, Lane 2: Avidin, Lane 3: Avidin, Py-Biotin and
 801 CoHA (Dark control), Lane 4-7: Avidin, Py-Biotin, and CoHA irradiated for 5, 10, 15, and 30 minutes,
 802 Lane 8: Avidin and Py-Biotin, Lane 9: Avidin and CoHA irradiated for 30 minutes. Reproduced with
 803 permission from the Journal of Photochemistry and Photobiology B.⁴⁴

805 4.2.2.3 Photochemical Studies with Py-Biotin and Avidin

806 Upon photoexcitation of pyrene at Py-Biotin's maximum absorption wavelength (342 nm), in
 807 the presence of CoHA, the cleavage of avidin should occur near the Py-biotin binding site. The protein
 808 solution (15 μM avidin), containing Py-biotin (15 μM) and CoHA (1 mM) in 50 mM Tris-HCl buffer,
 809 pH 7.0 (total volume 200 μL), was irradiated at 342 nm using a 150 W xenon lamp. A UV cutoff filter
 810 (WG-345; 78%T at 342 nm) was used to remove short wavelength radiation. Dark controls were

811 prepared under the same conditions, as described above, except that the solutions were protected from
812 light. All reaction mixtures were dried under reduced pressure and assayed on SDS/PAGE (Figure
813 11C). Two new photofragments were observed upon irradiation of avidin with Py-Biotin and CoHA
814 (Figure 11C)⁵ Yields of photoproducts increased steadily with irradiation time and reached a maximum
815 at 30 minutes, but the yields of the photoproducts were substantially less than noted with Py-Phe/BSA.
816 This outcome could be due to the substantially poor access of the probe to CoHA as inferred from
817 Figure 11B.

818 To test if Py-Biotin binds to avidin at the biotin binding site, competitive binding was studied
819 by mixing pure d-biotin with avidin/Py-Biotin before performing the photoreaction. If the binding sites
820 of d-biotin and Py-Biotin are the same, d-biotin should compete for the binding and may displace it. In
821 the inhibition study, the photoreactions were performed as above but d-biotin (60 mM) was mixed with
822 the protein solution for 15 minutes prior to addition of Py-biotin (15 mM) and CoHA (1 mM). The
823 solutions were then irradiated at 342 nm as described above and products analyzed by SDS/PAGE. The
824 Py-Biotin photoreaction was suppressed by biotin substantially (data not shown), supporting the idea
825 that the probe binds at the biotin binding site, in a competitive manner.⁵

826 **4.2.2.4 Sequencing Studies**

827 Amino acid sequencing analysis of the photochemical fragments from avidin/Py-Biotin
828 indicated that the peptide is cleaved between Thr77 and Val78, which is known to be in proximity to
829 the biotin binding site⁵. This was confirmed by docking studies where a unique binding site was
830 noticed, described below.

831 **4.2.3 Computational Modeling**

832 The docking results are shown in Figure 12, and Py-Biotin had a binding energy of -10
833 kcal/mol. Various residues show potential hydrogen bonding, specifically Asn12, Ser16, Tyr33, Thr35
834 and Asn118 with the probe. The distance between the pyrene chromophore (edge) to Thr77 was 10 Å,
835 which is in reasonable proximity for the photocleavage to occur at Thr 77 and Val 78.

Figure 12. Docking of Py-Biotin with Avidin and residues at the binding site are within good proximity to the observed cleavage site.

4.3. Squaraine Dyes

Squaraine dyes are a class of photostable organic molecules that have diverse applications including imaging, sensing and, nonlinear optics.^{27,27} These dyes typically have zwitterionic structures that are resonance stabilized.²⁷ The squaraine dyes discussed here are bis(2,4,6-trihydroxyphenyl) squaraine (D101)⁴⁵, bis(3,5-dibromo-2,4,6-trihydroxyphenyl) squaraine(D103), or bis(3,5-diiodo-2,4,6-trihydroxyphenyl) squaraine) D105 (Scheme 6).^{46,47} The halo-derivatives (D103 and D105), might undergo photodecomposition to generate the corresponding halogen radicals, which then could induce protein cleavage at the squaraine dye binding site. Thus, this class of dyes provided us a different avenue to test protein photocleavage reactions. Much like Py-Phe and its derivatives, changing the substituent on the ring systems changed the absorption and emission properties of the dye, and due to their high sensitivity to the environment, the three dyes differed significantly in terms of their interactions with proteins.

Scheme 6. Squaraine dyes under study (from left to right, D101, D103, and D105).

4.3.1 Photophysical studies

4.3.1.1 Absorption and Fluorescence Titrations

857 Squaraine dyes differ from Py-Phe and its derivatives in that they have “turn on”
 858 fluorescence.⁴⁸ The dyes themselves have a low fluorescence lifetime (<50 ps) because the dyes
 859 aggregate in solution, binding to protein has been suggested to break up the aggregates, relieving self-
 860 quenching and increasing their fluorescence intensities.

861 A. B. C.
 862 *Figure 13. (A) Absorption titration of D101 (1.1 μM in 2% v/v ethanol-water mixture, pH 8.4) with*
 863 *lysozyme (0 to 7.9 μM). (B) Fluorescence titration of 1.1 μM D101 (2% (v/v) ethanol) with lysozyme (0*
 864 *to 7.9 μM) in phosphate buffer pH 8.4, 575 nm excitation. (C) Scatchard plot obtained from the*
 865 *absorption titration shown in panel A, which indicated a binding constant of 3x10⁶/M.*

866
 867 When increased concentrations of lysozyme (0 to 7.9 μM) were titrated into a solution of D101
 868 (1.1 μM, in 2 v/v% ethanol-water mixture, pH 8.4), there has been substantial decrease in absorbance
 869 as well as fluorescence (Figure 13 A).^{28,3449} The interaction was further confirmed by constructing a
 870 binding isotherm using the Scatchard equation (eq. 1),³⁶ and the corresponding binding plot is shown
 871 in Figure 13C. Best fit to the data indicated a single binding site with a binding constant of 3x10⁶/M
 872 for D101.

873 Similar spectral changes were noted for D103 (5 μM in 2% ethanol-water mixture, pH 6.4) and
 874 D105 (1.4 μM, 2% v/v ethanol in water, pH 6.4) titrations with lysozyme (0 to 6.2 μM), as above, and
 875 the corresponding Scatchard plots obtained from the absorption titrations indicated binding constants
 876 of 1.0x10⁸/M and 2.0x10⁶/M for D103 and D105, respectively. Thus, the affinity was substantially
 877 higher for D103 than D101 or D105, but all three dyes indicated hypochromism and fluorescence
 878 quenching on binding to lysozyme.

879 Absorbance spectra of the squaraine dyes (30 μ M) mixed with BSA (30 μ M) were monitored.
 880 There have been immediate changes in the absorbance when the probes bind to the protein (in 10 mM
 881 sodium phosphate buffer, 50 mM NaCl, pH 7.4, Figure 15), but these changes continued further for up
 882 to 4 h, after which no substantial changes were detected. The peak wavelength of D101 blue shifted by
 883 13 nm to 582 nm, immediately after mixing. The shoulder at 500 nm also blue shifted approximately
 884 20 nm and all peaks decreased in intensity, as a function of incubation time (data not shown) Thus, the
 885 initial binding is followed by further interaction with BSA, which was not the case with the Py-Phe
 886 derivatives described above.

887 A. Figure 14. (A) The shift in absorbance of the squaraine dyes bound to BSA (D101-BSA pink solid,
 888 D103-BSA blue dashed, D105-BSA purple dotted lines) compared to those of the dyes in 10 mM
 889 phosphate 50 mM sodium chloride buffer pH 7.4 (D101 black solid, D103 black dashed, D105 black
 890 dotted lines). (B) Absorbance of 30 μ M D103 in the presence of 30 μ M BSA in 10 mM phosphate 50
 891 mM sodium chloride buffer pH 7.4 as a function of time. (C) Absorbance of 30 μ M D105 in the
 892 presence of 30 μ M BSA in 10 mM phosphate 50 mM sodium chloride buffer pH 7.4 as a function of
 893 time. Time 0 in these panels is the spectra immediately after mixing the dye and BSA, which is
 894 substantially different from that of the free dye.
 895

896
 897 The absorption peaks of D103 and D105 at $\sim$ 550 nm decreased in intensity while a new peak at
 898 620 nm appeared for both D103-BSA and D105-BSA samples (Figure 14 B, C). The 625 nm peak
 899 decreased at a faster rate than 550 nm peak. Dilution of the dye-BSA solutions did not restore the
 900 absorbance spectra to their original profiles, which suggested that the changes are not due to simple
 901 physical association, as seen with Py-Phe derivatives. Control experiments of the squaraine dyes
 902 dissolved in buffer under identical conditions revealed a decrease in absorbance similar to that

903 observed with BSA, which indicated an eventual chemical reaction of the dye with the solvent or the
 904 protein. These observations were further tested in CD and precipitation studies.

905 4.3.1.2 Circular Dichroism Studies

906 There has been no significant change in the deep UV CD of BSA (Figure 15A) when the dyes
 907 were titrated, which indicated no significant distortion of the protein structure upon probe binding.
 908 Binding of these probes to lysozyme also did not distort the protein structure to any significant extent,
 909 only small changes have been noted (Figure 15B). On the other hand, induced CD bands were noted
 910 upon binding of the squaraine dyes (30 μ M) to lysozyme (60 μ M, phosphate buffer), in the visible
 911 region, far from the UV CD bands of the protein (Figure 15C). That is, the binding places the
 912 chromophores in the asymmetric environment and this trigger induced CD in the transitions that
 913 correspond to the dye absorption bands in the visible region. Intense induced CD bands were noted for
 914 D103 and D105 but that of D101 has been very weak.

916 *Figure 15. (A) UV CD spectra of BSA-squaraine dyes, D101-BSA (purple), D103-BSA (blue) and*
 917 *D105-BSA (pink) and BSA (green) in phosphate buffer. Spectra of D10X in buffer are shown but could*
 918 *not be distinguished from that of the baseline. (B) UV CD spectra of lysozyme-squaraine dyes in*
 919 *phosphate buffer, D101-lysozyme (purple), D103-lysozyme (blue), and D105-lysozyme (pink) and*
 920 *those of free dyes in phosphate buffer, D101 (light purple), D103 (light blue) and D105 (light pink).*
 921 *(C) Induced CD of the squaraine dyes bound to lysozyme in the phosphate buffer, D101/lysozyme*
 922 *(pink), D103/lysozyme (purple) and D105/lysozyme (blue).*

923

4.3.1.3 Enzyme Inhibition and Binding Studies with Individual Amino Acids

Binding of the squaraine dyes to lysozyme could inhibit its activity, if the photoreagent binds at or near the active site of the enzyme. This possibility was tested by examining lysozyme-induced degradation of bacterial cells (*micrococcus lysodeikticus*) by reported methods.⁴⁹ Lysozyme activity was not inhibited by the addition of D101 or D103 but there has been significant inhibition by D105 (Figure 16B, pink). Thus, one concludes that D101 and D103 did not block the active site or bind to residues at the active site, but D105 is likely blocking access to the active site of the enzyme or reacted with active site residues, thus inhibiting enzyme activity to a substantial extent.

To test this hypothesis, we examined the reaction of several amino acids with the squaraine dyes by fluorescence spectroscopy (Figure 16A). As increasing amounts of cysteine were titrated into a solution of D101, a decrease in fluorescence emission at 600 nm was observed indicating interaction between D101 and cysteine. Covalent interaction was confirmed by separating the dye bound to the protein by acetone precipitation of lysozyme-D10X solutions. All three dyes are soluble in acetone and if the dye is covalently bound to the protein then acetone precipitation of the protein would not release the dye from the protein into the solvent. On the other hand, if the dye is only adsorbed onto the protein by non-covalent interactions, then it will be released into acetone solvent. Samples containing 1:1 mole ratio of lysozyme and the squaraine dyes were treated with acetone, incubated at -20°C for one hour then centrifuged to remove the protein, and the absorption spectra of the supernatant recorded. Control experiments were carried out where the dye solutions were treated in a similar fashion in the absence of the protein, and the corresponding spectra recorded. From these data, the dye concentrations in acetone were estimated and shown in Figure 16C.

Figure 16. (A) Fluorescence spectra of D101 with increasing concentrations of cysteine (0 μ M, (red) to 8.5 μ M, (purple)) in 2 % ethanol (v/v) in phosphate buffer, upon excitation at 560 nm. (B). Enzymatic activity of lysozyme monitored after dye binding, in phosphate buffer. Activities of lysozyme (no dye, black), lysozyme-D101 (purple), lysozyme-D103 (blue), and lysozyme-D105 (pink). (C). Concentrations of the squaraine dyes in supernatant after acetone precipitation, D101 (light purple), D101-lysozyme (dark purple), D103 (light blue), D103-lysozyme (dark blue), D105 (light pink) and D105-lysozyme (dark pink).

The acetone precipitation showed considerable drop in dye concentration in the acetone phase, after precipitation, for all the three dye samples (Figure 16C), and recovery of the dyes in the absence of the protein has been substantial. These data confirmed that the three squaraine dyes bind to lysozyme by covalent adduction to particular residues, and these could not be released by acetone precipitation of the protein. Thus, the protein-bound probes were tested for photocleavage reactions to identify the probe binding sites on the protein.

4.3.2 Photochemical Properties

4.3.2.1 SDS/PAGE

963 A mixture of lysozyme, CoHA and squaraine dyes in phosphate buffer were irradiated in RPR-
 964 100 photoreactor, equipped with visible light, for 30 min. The SDS/PAGE results are shown in Figure
 965 17 and there have been substantial presence of at least one photoproduct when all three reagents are
 966 present (lanes 4 and 8). However, the control lanes that did not contain the squaraine dye (lanes 5 and
 967 9) also indicated the same product band, which clearly indicated photocleavage due to CoHA. Indeed,
 968 we have previously reported the photocleavage of lysozyme by CoHA⁵⁰ and current experiments
 969 showed that the squaraine dyes did not cleave lysozyme under these conditions.

970

971 *Figure 17. Photocleavage studies of D101 and D103 with lysozyme in 10 mM sodium phosphate 50*
 972 *mM sodium chloride buffer pH 7.4 after irradiation with white light. Lane 1, molecular weight*
 973 *markers. Lanes 2 – 10 contain 15 μM lysozyme, lanes 3,4,7 and 8 contained 5 mM CoHA, lanes 3-4,*
 974 *and 6 contained 30 μM D101, lanes 7-8, and 10 contain 30 μM D103. Lanes 4-6 were irradiated for 6*
 975 *h, lanes 8-10 were irradiated for 12 h. Lanes 3 and 7 were protected from light. Photocleavage*
 976 *products of 11 and 5 kDa are present in lanes 4, 5, 8 and 9.*

977

978 A number of photoreagents have been systematically investigated for their interactions with a
 979 small set of proteins and their photoreactions have been examined in sufficient detail. By far, most of
 980 the probes examined here were successful in inducing protein photocleavage with the exception of the
 981 squaraine dyes, which did not photocleave lysozyme.

982 **5. Other Contributions to the Field of Protein Photocleavage**

983 A number of other groups around the world have extended these design features, subsequent to
 984 our initial reports of the protein photocleavage investigations. A few representative examples will be
 985 mentioned here, briefly, to demonstrate the potential of the above methodology but this is not a
 986 thorough review of other work. Photoexcitation of anthraquinone and 2-hydroxymethylantraquinone
 987 (5-150 μM) with BSA (15 μM) produced photocleavage.⁵¹ Samples were irradiated at 365 nm, in 20%
 988 acetonitrile 50 mM Tris-HCl, pH 7.0. SDS/PAGE showed no specific product bands but cleavage was

random. Anthraquinone weakly cleaved BSA while 2-hydroxymethylantraquinone significantly cleaved BSA but no yields were reported not cleavage sites identified. Further experiments with lysozyme (15 μM) and derivatives (anthraquinone-triethylene glycol and anthraquinone-carbohydrate, 5-150 μM) also resulted in photoreactions via reactive oxygen species generated during the reaction.

Photoexcitation of Phenol Red-modified 5,10,15,20-tetraphenylporphyrin (R-TPP, 25 μM) at 420 nm cleaved both BSA and lysozyme (25 μM) in 3:1 PBS/DMS via singlet oxygen, but photoreaction yields and protein cleavage sites were not reported.⁵² The phenol red moiety in the probe was suggested to contribute to the binding affinity, amphiphilic character, and suppress aggregation. Binding constants of R-TPP reported with BSA was $8.49 \times 10^4 \text{ M}^{-1}$ and with lysozyme has been $2.11 \times 10^4 \text{ M}^{-1}$. This was further expanded with derivatives of rhodamine-3-acetic acid (RhD-TPP and RhDCOOH-TPP)⁵³ to cleave BSA (2 μM) using radiation $<420 \text{ nm}$. Binding constants for both rhodamine derivatives were reported to be $4.67 \times 10^4 \text{ M}^{-1}$ and $4.46 \times 10^4 \text{ M}^{-1}$, respectively.⁵³

An interesting example is the protein photocleavage of the transcription factor, ER- α by 2-Phenylquinoline, 2-phenylquinoline-estradiol and 2-phenylquinoline-4-hydroxytamoxifen.⁵⁴ The estradiol and tamoxifen are biologically active for breast cancer or osteoporosis treatments. 2-phenylquinoline degraded proteins with long wavelength UV light, under pH neutral conditions. Excitation at 365 nm of the quinoline probes generates a reactive oxygen species and led to non-specific photocleavage of ER- α . But the fragments were too small in quantity to be detected by MALDI-TOF or SDS-PAGE.⁵⁴ Upon incubation with MCF-7 cells and irradiation at 365 nm, both quinoline derivatives inhibited cell growth in a dose dependent manner.

6. Conclusions

A number of small organic molecules have been designed, synthesized and tested for their binding to proteins, which indicated several examples where the photoreaction has been successfully induced. The binding affinities, however, depended on probe structure in a complex manner, and could not be rationalized by any simple model. While their affinities differed, they all responded substantially on binding to the protein, in terms of their photophysical properties. Protein binding resulted in substantial changes in the absorption and fluorescence spectra, and the data have been quantified using Scatchard equation. In all the cases examined here, except for Py-Phe/BSA, all systems indicated a single binding site with moderate to high affinities. Binding to the protein also buried the chromophore in the protein matrix and its access to the solvent was successfully probed in

1019 fluorescence quenching studies by CoHA. Furthermore, the degree of protection offered by the protein
1020 against quenching with CoHA roughly correlated inversely with the yield of the photocleavage
1021 reaction, yields with lysozyme being higher than with BSA. Thus, some access to the bound
1022 chromophore by CoHA was essential to initiate the photoreaction.

1023 The newly created N-terminus of the photoproduct was amenable to conventional sequencing
1024 studies, which is an important result with ramifications about the application of these photoreagents for
1025 biochemical studies. However, the newly created C-terminus was not sequenceable, but these
1026 constraints helped us in narrowing down the possible mechanistic schemes that could be devised.
1027 Another significant observation was that many of the photoreagents shared the same cleavage site on
1028 BSA and lysozyme, with some exceptions. This could be because they all had the large hydrophobic
1029 pyrenyl group but the side chain played an important role. Longer chains allowed the probe to access
1030 residues that are farther and new cleavage sites were noted. Along the same lines, the attachment of
1031 biotin in the side chain allowed Py-Biotin to be directed to the biotin binding site and this is likely due
1032 to the higher affinity of biotin to its binding site on avidin when compared to that of the pyrenyl
1033 moiety.

1034 Computer docking studies, conducted recently, provided strong support for the photocleavage
1035 and sequencing studies. The docked structures indicated good proximity of the photoreagent to the
1036 observed cleavage site, and the experimental data have validated the success of the computer
1037 modeling. This indeed, is surprising, given the uncertainties involved in the experiment as well as in
1038 the computational modeling. In future studies, computer modeling may be used in an iterative manner,
1039 to target specific sites on a given protein, followed by experiments to verify if a predicted site could be
1040 hit with a designed probe. We are not there yet, but with increased computational power and the
1041 availability of reliable and sophisticated algorithms should make this a viable strategy to photocleave
1042 proteins at desired sites.

1043 In summary, the photoreagent design should consider several factors, such as potential binding
1044 sites on the target protein, desired structural features of the photoreagent to bind at the binding site and
1045 the excited state properties of the chromophore as well as the quencher. Attachment of the quencher to
1046 the chromophore would be the next step and this would remove the requirement that the probe be
1047 accessible to the solvent for efficient quenching to initiate the photochemistry. The excited state need
1048 to live long enough to induce the desired reaction at the protein binding site but it also should have the

necessary chemical reactivity. CoHA played an important role in the examples presented here, where it generated a long lived radical intermediate from the pyrenyl chromophore, which successfully induced the photocleavage of the protein backbone. There could be other possible intermediates that would cleave the protein backbone, selectively and efficiently. This type of photochemical control of biological macromolecular chemistry is likely to be interesting in the near future.

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