ChemComm

Large chiral discrimination of a molecular probe by bovine serum albumin

Challa V. Kumar,* Apinya Buranaprapuk† and Ho Chou Sze

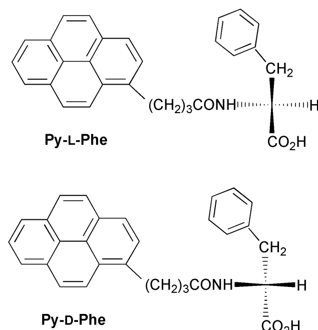
Department of Chemistry, U-60, University of Connecticut, 55 N. Eagleville Road, Storrs, CT 06269-3060, USA. E-mail: c.v.kumar@uconn.edu

Received (in Columbia, MO, USA) 21st August 2000, Accepted 15th December 2000
First published as an Advance Article on the web 23rd January 2001

A phenylalanine-derived fluorescent probe shows high enantioselectivity for binding to bovine serum albumin; the L-isomer binds nearly one hundred times better than the D-isomer, which suggests the tight fitting of the L-isomer to the binding site.

Although chiral discrimination of substrates by enzymes and other biological macromolecular systems is well known, the design of chiral molecules that can bind to proteins with a high enantioselectivity is challenging.[1] Enantioselective binding of dansyl amino acids, drugs, and metal complexes to bovine serum albumin (BSA), chymotrypsin, and other proteins has been reported, although the selectivity is often low (1.15 to 3).[2] Despite the low selectivity, chiral biochromatography with BSA has been demonstrated to be useful in separating the optical isomers of drugs, amino acids, and small molecules, thereby, indicating the utility of such recognition in a variety of applications.[3] The observed enantiomeric selectivity was explained based on the steric, hydrophobic and hydrogen-bonding interactions between the ligand and the host.[4] The design of ligands that bind tightly to sites that can accommodate only one isomer but not the other, with selectivities >10, therefore, is challenging.

The design of pyrene labeled peptides that bind to proteins and induce site specific photocleavage of the protein backbone (protein scissors) is of current interest.[5] *N*-[4(1-pyrene)butyroyl]-L-phenylalanine (Py-L-Phe), for example, induces site specific photocleavage in BSA between residues Leu 346 and Arg 347, and in lysozyme the cleavage occurs between residues Trp 108 and Val 109.[5c] We now report that Py-L-Phe and its optical isomer, Py-D-Phe, are discriminated by BSA in a 100 : 1 ratio (the ratio of the corresponding binding constants). These observations indicate the importance of the single asymmetric center present in Py-Phe for recognition between ligand and the protein. The chiral discrimination is reflected in the photophysical properties of the protein-bound probes. The protein-bound ligand spectra exhibit contrasting differences between the isomers, indicating the sensitivity of these properties on the chiral nature of the probe microenvironment in the protein.



Py-D-Phe was synthesized by reacting the methyl ester of D-phenylalanine with 4(1-pyrene)butyric acid in tetrahydrofuran using *N,N′*-dicyclohexyl carbodiimide (DCC) as the coupling agent in a procedure analogous to the synthesis of the corresponding L-isomer.[5] Mild hydrolysis of the methyl ester (1 M HCl, 12 h, room temp.) followed by column chromatography on silica gel (elution with 20 : 5, chloroform to methanol) resulted in Py-D-Phe in 60% yield. The product was characterized from UV–VIS, fluorescence, circular dichroism, ^{1}H NMR and mass spectral data. ^{1}H NMR (400 MHz, d$_6$-DMSO) δ 7.8–8.2 (9H), 7.1 (5H), 4.4 (1H), 3.1 (2H), 1.8–2.1 (6H); MS data (FAB): *m/z* 436 (MH$^+$).

The absorption spectrum of the pyrenyl chromophore in the near UV region (300–360 nm) is sensitive to the binding of the probe to the protein. Addition of BSA (0, 0.5, 1, 1.5, 2, 4, and 8 μM) to Py-D-Phe (10 μM) resulted in dramatic changes in the probe absorption spectrum (Fig. 1). The vibronic band positions are shifted to longer wavelengths as the protein is titrated into the solution, and an isosbestic point (point of constant absorbance) is clearly visible. Such isosbestic behavior is strongly indicative of two distinct chromophores, and we assign these to the bound and free chromophores. A net increase in the absorbance at the 0–0 band (hyperchromism) with protein concentration is also evident in the spectra. In contrast to these results, extensive hypochromism (decrease in absorbance) was observed when Py-L-Phe was titrated with BSA, under similar conditions.

The above absorption titration data were analyzed to construct binding isotherms, using the Scatchard equation,[5] and the corresponding binding constant is 5.3×10^5 dm^3 mol^{-1}. This value is two orders of magnitude less than the corresponding binding constant for the L-isomer (6.7×10^7 dm^3 mol^{-1}).[5] Thus, Py-L-Phe binds nearly 100 times better than Py-D-Phe. The large discrimination between the two chiral isomers is surprising, and it is much greater than the enantoselectivities observed for the binding of gossypol, warfarin, dansyl amino acids and chiral fatty acid analogs to BSA (discussed below). The large differences in the binding constants between the optical antipodes strongly suggest the intimate interaction of the probe with the protein in which only one of the two isomers fits snugly in the binding site. Such intimate interaction with the surrounding protein matrix is expected to result in differences in the spectral properties of the protein-bound chromophores.

The microenvironments of the protein-bound isomers, their access to the aqueous phase, and solvent exposure are expected

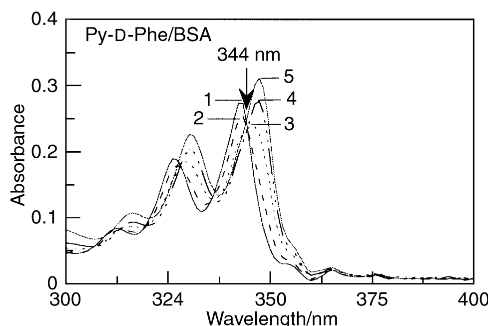


Fig. 1 Absorption spectra of Py-D-Phe (10 μM) (1 cm path length) with increasing concentrations of BSA: (0, 0.5, 2.0, 4.0 and 8.0 μM). The red shift (4 nm) of the peak positions and the isosbestic points at 344, 328, and 315 nm are evident in the spectra.

† Current address: Department of Chemistry, Faculty of Science, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand.

DOI: 10.1039/b007352k

This journal is © The Royal Society of Chemistry 2001

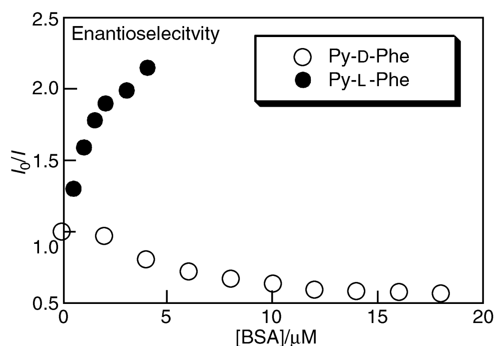


Fig. 2 Plot of the fluorescence intensities (I_0/I) of Py-D-Phe (2 μM, open circles) and Py-L-Phe (2 μM, filled circles) recorded at increasing concentrations of BSA. Note that larger concentrations of BSA are needed to saturate binding of Py-D-Phe. Both samples were excited at 344 nm.

to be different. These details are examined in fluorescence experiments. The protein–probe mixtures are excited at 344 nm (isosbestic point) and the probe emission was monitored at increasing concentrations of the protein. The ratio of initial intensity (I_0) to that in the presence of BSA (I) is plotted as a function of BSA concentration (Fig. 2). The fluorescence intensity of the D-isomer increases initially with protein concentration, and the ratio, I_0/I, reaches a plateau at higher protein concentrations. The fluorescence from the L-isomer, in contrast, is strongly quenched by BSA, and the corresponding plot shows an accidental near-mirror image behavior. These results highlight the differences in the local environment surrounding the fluorophore when the two isomers bind to BSA.

The accessibility of the two isomers to the solvent was probed using the fluorescence quencher, hexamminecobalt(III) chloride. The extent of protection offered by the protein can be readily distinguished in these simple experiments. The emission from the BSA-bound D-isomer was enhanced by the addition of CoHA (Fig. 3) whereas for the L-isomer the emission was quenched, confirming the differences in the binding behavior of the two optical antipodes shown in Figs. 1 and 2.

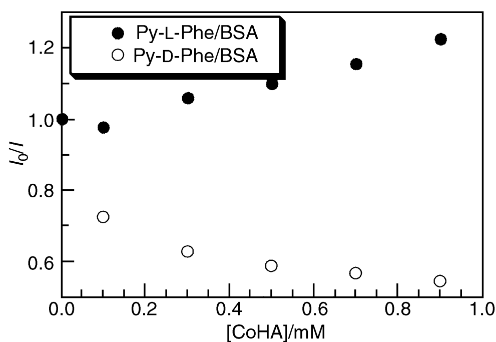


Fig. 3 Chiral selectivity for the quenching of fluorescence emission from the Py-D-Phe (open circles) and Py-L-Phe (filled circles) bound to bovine serum albumin by hexamminecobalt(III) chloride.

The large difference in the affinities of the optical antipodes arising due to a single chiral center is unexpected. For example, the binding of dansyl labeled amino acids to BSA shows only a 3:1 discrimination between the corresponding D- and L-isomers.[2] Treatment of racemic potassium tris(oxalato)cobaltate(III) with BSA resulted in the enrichment of the Δ isomer with an enantiomeric excess of 18%[6] and only minor differences were reported for the binding of the ketoprofen enantiomers to BSA.[7] The first report on the chiral recognition by a *de novo* designed peptide using D-norleucine derivative was reported to be 2:1,[8] and (*R*)-warfarin was retained to a greater extent on an immobilized BSA column than (*S*)-warfarin[9] while no enantioselectivity was observed for the binding of gossypol to BSA.[10] The D-amino acids, on a liquid chromatography column with immobilized BSA as the chiral phase, eluted at different times, but no information on the relative binding affinities of the isomers is reported. Preferential binding of L-phenylalanine to BSA was observed in ultrafiltration experiments,[10b] and in all the above examples, the observed selectivity was only moderate. The larger discrimination observed with Py-Phe, compared to the naphthyl or the dansyl amino acids, can be attributed to the increased hydrophobic surface area of the pyrenyl chromophore. Larger, rigid, hydrophobic surfaces of correct configuration can interact better with the protein, while a wrong isomer will interact poorly, thus widening the gap between the two binding constants.

The chiral center present in Py-Phe is five atoms away from the pyrenyl chromophore and yet it dramatically influences the binding behavior/spectral properties of the pyrenyl chromophore. Both isomers may bind at the same site or may bind at different sites on BSA. These two models can be distinguished in photocleavage experiments in which the pyrenyl probe can be activated with light to cleave the protein backbone at the probe binding site. Such experiments are in progress. Hydrophobic burial of Py-L-Phe at domain II, subdomain C of BSA at residues 346 and 347, was indicated from the photocleavage experiments from this laboratory, and current results are consistent with site specific binding of D and L isomers of Py-Phe to BSA.[5] The marked differences observed in the spectral properties of the bound enantiomers are surprising, and they are primarily due to differences in the residues that line the binding cavity. These results imply that the location of the pyrenyl chromophore, the photoactive moiety used for protein cleavage, is different for the two enantiomers.

Such differences in the binding environment will be exploited in photocleavage experiments to direct the photochemical reagents to different sites on proteins. The large differences in the spectral properties of Py-Phe isomers, in addition, provide a signal transduction mechanism for the translation of the chiral recognition information into an easily measurable spectroscopic quantity for application in chiral biosensors.

Financial support of this work by the National Science Foundation (DMR-9729178), and the sponsors of the Petroleum Research Fund, are gratefully acknowledged.

Notes and references

1 C. J. Suckling, *Enzyme Chemistry: Impact and Applications*, Chapman and Hall, London, 2nd edn., 1990; A. Lehninger, D. L. Nelson and M. M. Cox, *Principles of Biochemistry*, Worth, New York, 1993; F. H. Westheimer, H. Fisher, E. E. Conn and B. Vennesland, *J. Am. Chem. Soc.*, 1951, **73**, 2043.
2 Y. Abe, S. Fukui, Y. Koshiji, M. Kobayashi, T. Shoji, S. Sugata, H. Nishizawa, H. Suzuki and K. Iwata, *Biochim. Biophys. Acta*, 1999, **1433**, 188; A. Haque and J. T. Stewart, *J. Liq. Chromatogr. Relat. Technol.*, 1998, **21**, 2675; C. J. Stefan, M. Ubbink, G. W. Canters and H. P. J. M. Dekkers, *J. Phys. Chem.*, 1996, **100**, 17 957; G. Felix and V. Descorps, *Chromatographia*, 1999, **49**, 595; Y. Yan and M. L. Myrick, *Anal. Chem.*, 1999, **71**, 1958.
3 S. Allenmark and S. Andersson, *Chirality*, 1992, **4**, 24; T. Cserhati and E. Forgacs, *Int. J. Bio-Chromatogr.*, 1999, **4**, 203; L. Lepri, V. Coas and M. Del Bubba, *J. Planar, Chromatogr.-Mod., TLC*, 1999, **12**, 221; T. Kitae, T. Nakayama and K. Kano, *J. Chem. Soc., Perkin Trans. 2*, 1998, 207.
4 K. B. Lipkowitz, *Acc. Chem. Res.*, 2000, **33**, 555.
5 (*a*) A. Buranaprapuk, C. V. Kumar, S. Jockusch and N. J. Turro, *Tetrahedron*, 2000, **56**, 8311; (*b*) C. V. Kumar and A. Buranaprapuk, *J. Am. Chem. Soc.*, 1999, **121**, 4262; (*c*) C. V. Kumar, A. Buranaprapuk, G. J. Opiteck, M. B. Moyer, S. Jockusch and N. J. Turro, *Pro. Natl. Acad. Sci.*, 1998, **95**, 10 361; (*d*) C. V. Kumar and A. Buranaprapuk, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 2085.
6 T. Taura, *Inorg. Chim. Acta*, 1996, **252**, 1.
7 M. Levi and M. Zandomeneghi, *Gazz. Chim. Ital.*, 1966, **126**, 599.
8 K. S. Broo, H. Nilsson, J. Nilsson and L. Baltzer, *J. Am. Chem. Soc.*, 1988, **120**, 10 287.
9 G. Massolini, A. F. Aubry, A. McGann and I. W. Wainer, *Biochem. Pharmacol.*, 1993, **46**, 1285.
10 (*a*) D. S. Sampath and P. Balaram, *Biochim. Biophys. Acta*, 1986, **882**, 183; (*b*) A. Higuchi, T. Hashimoto, M. Yonehara, N. Kubota, K. Wanatabe, S. Uemiya, T. Kojima and M. Hara, *J. Membr. Sci.*, 1997, **130**, 31.

Typeset and printed by Black Bear Press Limited, Cambridge, England