รายงานวิจัยฉบับสมบูรณ์

การออกแบบและสังเคราะห์โมเลกุลชนิดใหม่เพื่อศึกษาการจับและตัดโปรตีนที่ตำแหน่งจำเพาะ

Design and synthesis of novel reagent for specific binding and cleavage of proteins

โดย รองศาสตราจารย์ ดร. อภิญญา ชัยวิสุทธางกูร

เมษายน พ.ศ. 2556

สัญญาเลขที่ RSA5280020

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ภาควิชาเคมี คณะวิทยาศาสตร์

มหาวิทยาลัยศรีนครินทรวิโรฒ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง “**การออกแบบและสังเคราะห์โมเลกุลชนิดใหม่เพื่อศึกษาการจับและตัดโปรตีนที่ตำแหน่งจำเพาะ (Design and synthesis of novel reagent for specific binding and cleavage of proteins)”** ได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย ตามสัญญาเลขที่ **RSA5280020** ระยะเวลาดำเนินการ 3 ปี 10 เดือน ตั้งแต่วันที่ 2 มีนาคม 2552 ถึงวันที่ 31 ธันวาคม 2555 ผู้รับทุนขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย ที่ให้การสนับสนุนอย่างเต็มที่ทำให้โครงการวิจัยสำเร็จลุล่วงไปได้ และขอขอบคุณบุคคลต่อไปนี้ที่มีส่วนสำคัญให้โครงการสำเร็จได้แก่ ผศ.ดร. ธีรยุทธ ลิ่วพรเจริญวงศ์ (ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ) ที่ให้ความช่วยเหลือในการสังเคราะห์สารเคมี ขอขอบคุณ ศ.ดร. อภิชาติ สุขสำราญ ที่ให้ความอนุเคราะห์การวิเคราะห์ทาง Mass spectroscopy และขอขอบคุณนิสิตคือ น.ส.ปิ่นปินัทธ์ มะลัยแก้ว น.ส.สุดารัตน์ เย็นใจ และ น.ส.เบญจวรรณ จิตยุติ (ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ) และ รวมทั้งภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ ในฐานะสถาบันต้นสังกัดที่ให้ความสนับสนุนและอำนวยความสะดวกในโครงการวิจัย

บทคัดย่อ

รหัสโครงการ: RSA5280020

ชื่อโครงการ: การออกแบบและสังเคราะห์โมเลกุลชนิดใหม่เพื่อศึกษาการจับและตัดโปรตีนที่ตำแหน่งจำเพาะ

ชื่อนักวิจัย และสถาบัน: รองศาสตราจารย์ ดร. อภิญญา ชัยวิสุทธางกูร
ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

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โครงการวิจัย:

การศึกษานี้เป็นงานวิจัยพื้นฐานเพื่อการประยุกต์ใช้ปฏิกิริยาทางเคมีแสงในการศึกษาปฏิกิริยาในระดับโมเลกุลขนาดใหญ่ (macromolecule) เช่น โปรตีน และ DNA เพื่อการศึกษาถึงโครงสร้างของตำแหน่งที่มีการจับของลิแกนด์ในโมเลกุลขนาดใหญ่ โดยออกแบบโมเลกุลที่ประกอบด้วยหมู่ฟังก์ชันสองหมู่คือ หมู่ไพรีนและหมู่ที่มีความจดจำจำเพาะ (เช่น สับสเตรทของเอนไซม์) หมู่ที่มีความจดจำจำเพาะจะทำหน้าที่นำโมเลกุลสังเคราะห์นี้ไปยังตำแหน่งที่จำเพาะบนโปรตีนเป้าหมาย ในงานวิจัยนี้ได้ทำการสังเคราะห์โมเลกุลชนิดใหม่คือ d-biotinyl-1(1-pyrene) methylamide (Py–biotin) โดยการเชื่อมต่อ d-biotin กับหมู่เอมีนของ 1(1-pyrene)methylamine hydrochloride เพื่อศึกษาการจับและตัดโปรตีน avidin และ streptavidin จากการศึกษาทาง spectroscopy พบว่ามีการเปลี่ยนแปลงของ absorption spectrum ของ Py-biotin เมื่อผสม avidin และ Py-biotin เข้าด้วยกัน และเมื่อนำ avidin และ Py-biotin มาฉายแสงที่ความยาวคลื่น 342 nm โดยมี cobalt(III) hexammine trichloride (CoHA) เป็นตัวรับอิเล็กตรอน พบว่าโปรตีนถูกตัดที่ตำแหน่ง Thr 77 และ Val 78 โดยคาดว่ากลไกของปฏิกิริยาเกิดผ่าน pyrene cation radical

นอกจากนี้ ผู้วิจัยยังได้ศึกษาปฏิกิริยาการตัดโปรตีนโดยใช้สารประกอบเชิงซ้อนโมลิบดินัม ซึ่งยังไม่เคยมีรายงานการตัดโปรตีนด้วยแสงโดยใช้สารประกอบเชิงซ้อนโมลิบดินัมมาก่อน โดยการสังเคราะห์สารประกอบเชิงซ้อนโมลิบดินัมชนิดใหม่ ที่มีกรดอะมิโนเป็นลิแกนด์ เนื่องจากกรดอะมิโนเป็นสับสเตรทของเอนไซม์บางชนิด ในงานวิจัยนี้ได้ทำการสังเคราะห์ $MoO(O_2)_2$(α-leucine) (H_2O) เพื่อศึกษาการตัดโปรตีนที่ตำแหน่งจำเพาะโดยการกระตุ้นด้วยแสง และใช้ pepsin เป็นโปรตีนตัวอย่าง จากการทดลองเมื่อฉายแสงที่ความยาวคลื่น 320 หรือ 340 nm เป็นเวลา 10-30 นาที ไปยังสารละลายผสมของ pepsin/$MoO(O_2)_2$(α-leucine)(H_2O) พบการตัดโปรตีนเกิดขึ้นที่ตำแหน่งระหว่าง Leu 112 และ Tyr 113 และพบว่าประสิทธิภาพการตัดโปรตีนที่ความยาวคลื่น 340 nm จะดีกว่าที่ความยาวคลื่น 320 nm และมีประสิทธิภาพมากขึ้นเมื่อใช้เวลาฉายแสงนานขึ้น นอกจากนี้การตัดโปรตีนจะถูกยับยั้งเมื่อเติมเอทานอลลงในสารละลายผสมของ pepsin/$MoO(O_2)_2$(α-leucine) (H_2O) ดังนั้น กลไกของปฏิกิริยาอาจเกิดขึ้นโดยมี hydroxyl radical เป็น intermediate เนื่องจาก

เอทานอลจะยับยั้งการเกิดปฏิกิริยาของ hydroxyl radical งานวิจัยนี้จะเป็นงานวิจัยพื้นฐานในการออกแบบโมเลกุลสังเคราะห์ชนิดใหม่ที่สามารถใช้เป็น metal-footprinting ในการศึกษา metal binding sites ในโปรตีนได้อีกด้วย

คำหลัก: photocleavage, pepsin, molybdenum complex, avidin, biotin, protein-metal interaction, pyrene

Abstract

Project Code: RSA5280020

Project Title: Design and synthesis of novel reagent for specific binding and cleavage of proteins

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Project Period: 2 March 2009 – 31 December 2012

Project Description:

The binding of small molecules (ligands) to proteins is an important event in many biological processes. One strategy to locate ligand binding sites on proteins is to attach a photochemical reagent to the ligand. Such an approach requires the design of photochemical reagents that are capable of binding proteins at specific sites. In this study, a new pyrenyl probe, d-biotinyl-1(1-pyrene)methylamide (Py–biotin) was designed and synthesized by coupling of d-biotin to 1(1-pyrene)methylamine hydrochloride. Binding studies and site-specific photocleavage of avidin and streptavidin by Py–biotin were demonstrated. Red shifts of the absorption peak positions of the pyrenyl chromophore followed by hyperchromism were observed upon binding to avidin. The photocleavage of avidin was achieved when a mixture of the protein, Py–biotin, and an electron acceptor, cobalt(III) hexammine trichloride (CoHA), was irradiated at 342 nm. N-terminal sequencing of the peptide fragments indicated a cleavage site of avidin between Thr 77 and Val 78. The formation of pyrenyl cation radical resulted from quenching of pyrene excited state by CoHA is expected to play the important role in the photoreaction.

In addition, the photocleavage of protein by a molybdenum complex is also demonstrated in this report. A molybdenum(VI) peroxo α-amino acid complex, $MoO(O_2)_2$(α-leucine) (H_2O), was synthesized and used for site-specific cleavage of porcine pepsin, a model protein. Photocleavage of pepsin by $MoO(O_2)_2$(α-leucine)(H_2O) was achieved upon irradiation of the pepsin/$MoO(O_2)_2$(α-leucine)(H_2O) mixture by UV light (320 and 340 nm) for 10-30 minutes. No cleavage was observed in the absence of $MoO(O_2)_2$(α-leucine)(H_2O) or the light. The photocleavage yield increased with irradiation time. N-terminal sequencing of the cleaved fragments indicated the assigned cleavage site to

Leu(112)-Tyr(113). The cleavage reaction was quenched by ethanol. Therefore, hydroxyl radicals may be involved in the reaction and responsible for the cleavage of the protein. This is the first demonstration of the successful photocleavage of proteins by a molybdenum complex. This observation can provide a new approach for the photochemical footprinting of metal binding sites on proteins.

Keywords: photocleavage, pepsin, molybdenum complex, avidin, biotin, protein-metal interaction, pyrene

หน้าสรุปโครงการ (Executive Summary)
ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา ปี 2552

ชื่อโครงการ **การออกแบบและสังเคราะห์โมเลกุลชนิดใหม่เพื่อศึกษาการจับและตัดโปรตีนที่ตำแหน่งจำเพาะ**

การดำเนินการ

✓ ได้ดำเนินงานตามแผนที่วางไว้

☐ ได้ดำเนินงานล่าช้ากว่าแผนที่วางไว้

☐ ได้เปลี่ยนแผนงานที่วางไว้ดังนี้

วัตถุประสงค์

สังเคราะห์โมเลกุลชนิดใหม่เพื่อศึกษาปฏิกิริยาการจับระหว่างโมเลกุลขนาดเล็กกับโปรตีนที่เหมาะสม และศึกษาปฏิกิริยาการตัดโปรตีนที่ตำแหน่งจำเพาะอย่างมีประสิทธิภาพ เพื่อเป็นพื้นฐานสำหรับงานวิจัยอื่นๆ ที่เกี่ยวข้อง และเพื่อการประยุกต์ใช้ในการออกแบบยาที่สามารถจับกับเอนไซม์และยับยั้งการทำงานของเอนไซม์

รายละเอียดผลการดำเนินงานของโครงการ

สิ่งที่ได้ดำเนินการ

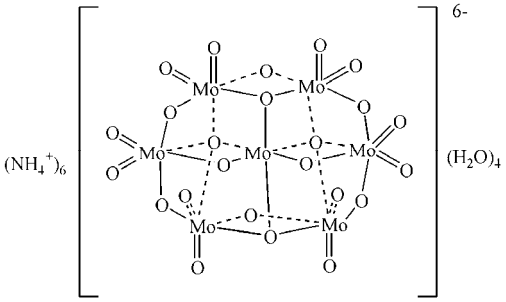
ผู้วิจัยได้ทำการสังเคราะห์อนุพันธ์ไพรีนชนิดใหม่ คือ d-biotinyl-1(1-pyrene) methylamide (Py-biotin) และนำมาศึกษาการจับกับโปรตีนเป้าหมายคือ avidin ซึ่งเป็นที่ทราบกันดีว่า biotin เป็นสับสเตรท ของ avidin (และ straptavidin) โดยสามารถจับกับ avidin ด้วยค่า binding constant สูงถึง 10^{15} dm^3mol^{-1} ในงานวิจัยนี้ใช้เทคนิคทาง UV-VIS และ Fluorescence spectroscopy ในการศึกษาปฏิกิริยาระหว่าง Py-biotin และ avidin พบการเปลี่ยนแปลงของ absorption spectrum และ fluorescence spectrum ของ Py-biotin เมื่อ Py-biotin จับกับ avidin และสามารถนำ Py-biotin ไปทดสอบการตัดโปรตีนได้สำเร็จ โดยทำการฉายแสงที่ความยาวคลื่น 342 nm ซึ่งเป็นช่วงความยาวคลื่นที่ Py-biotin ดูดกลืนแสงได้ดี โดยมี cobalt(III) hexamine trichloride (CoHA) เป็น electron acceptor ซึ่งการตัดโปรตีนนี้ทำให้ได้เพปไทด์ย่อย 2 แถบจากการตรวจสอบด้วยวิธี SDS-PAGE โดยเพปไทด์ย่อยที่เกิดจากการตัดมีมวลโมเลกุลประมาณ 9 และ 5 kDa จากการทำ western transfer จาก gel ไปยัง PVDF membrane เพื่อวิเคราะห์หาลำดับกรดอะมิโนของสายเพปไทด์ที่ได้จากการตัดโปรตีน (โดยส่งไปวิเคราะห์ที่ Midwest Analytical, Inc., MO, USA) สามารถหาลำดับกรดอะมิโนจากปลายด้าน N ของเพปไทด์ที่ได้จากการตัดได้ พบว่าการตัด avidin เกิดขึ้นระหว่าง Thr(77) และ Val(78)

ผลงานนี้ได้รับการตีพิมพ์ดังนี้

Malaikaew, P.; Svasti, J.; Kumar, C. V.; *Buranaprapuk, A.* (2011) Photocleavage of avidin by a new pyrenyl probe. *J. Photochem. Photobiol. B*, 103, 251–255.

นอกจากนี้ผู้วิจัยได้ทำการสังเคราะห์อนุพันธ์ไพรีนชนิดใหม่อีกชนิดหนึ่ง คือ d-gluco pyranosyl-1(1-pyrene) methylamide (Py-Glc) เพื่อศึกษาปฏิกิริยาระหว่างอนุพันธ์ไพรีนชนิดใหม่นี้กับโปรตีนตัวอย่าง ได้แก่ lysozyme พบว่า Py-Glc ตัดโปรตีน lysozyme เมื่อนำสารละลายผสมระหว่างโปรตีน Py-Glc และ CoHA (electron acceptor) มาฉายแสงที่ความยาวคลื่น 349 nm พบการตัดโปรตีนเกิดขึ้นได้แถบเพปไทด์ใหม่ 2 แถบที่มีมวลโมเลกุลประมาณ 10 kDa และ 4 kDa นอกจากนี้ผู้วิจัยได้ทดลองศึกษาการตัดโปรตีนชนิดอื่น ได้แก่ α-casein และ lectin โดยทำการทดลองเช่นเดียวกับการศึกษาการตัด lysozyme ไม่พบการตัดโปรตีนเหล่านี้เกิดขึ้น

เนื่องจากโมเลกุลของ Py-Glc มีปัญหาเรื่องการละลายในสารละลายบัฟเฟอร์ และสารละลาย Py-Glc ไม่ค่อยเสถียร ค่าการดูดกลืนแสงของสารละลาย Py-Glc ลดลงในเวลาอันรวดเร็ว ผู้วิจัยจึงได้เปลี่ยนการสังเคราะห์เป็นโมเลกุลชนิดใหม่ โดยใช้สารประกอบเชิงซ้อนโมลิบดินัม และยังไม่มีรายงานการใช้โมลิบดินัมในการตัดโปรตีนอย่างจำเพาะและมีประสิทธิภาพโดยการกระตุ้นปฏิกิริยาด้วยแสงมาก่อน โดยในปี 2012 ผู้วิจัยได้มีรายงานตีพิมพ์การใช้ไอออนลบของโลหะโมลิบดินัม $\{[Mo_7O_{24}]^{6-}\}$ จาก Ammoniumheptamolybdate tetrahydrate $[(NH_4)_6Mo_7O_{24} \cdot 4H_2O]$ ในการตัดโปรตีน pepsin โดยการกระตุ้น $[Mo_7O_{24}]^{6-}$ ด้วยความร้อนที่อุณหภูมิ 37 $^{\circ}C$ ในสารละลายบัฟเฟอร์ pH 7.0 และเนื่องจากโมเลกุลของ $[Mo_7O_{24}]^{6-}$ เป็น cluster ดังแสดงในภาพด้านล่าง ดังนั้นจึงใช้ความเข้มข้นของ $[Mo_7O_{24}]^{6-}$ เพียง 0.125 mM ในการตัดโปรตีน pepsin และปฏิกิริยาเกิดขึ้นหลังจากบ่มสารละลายผสมเพียง 2 ชั่วโมง



โครงสร้างของ Ammoniumheptamolybdate tetrahydrate $[(NH_4)_6Mo_7O_{24} \cdot 4H_2O]$

(Yenjai, S. et al **(2012)** Selective cleavage of pepsin by molybdenum metallopeptidase. *Biochem. Biophys. Res. Comm.*, **419**, 126–129.)

จากผลการทดลองพบว่า pepsin ถูกตัดและเกิด band ใหม่ของเพปไทด์ที่ได้จากการตัด 3 band โดยการเกิดปฏิกิริยาไม่จำเป็นต้องใช้ reducing agent และคาดว่ากลไกการเกิดปฏิกิริยาเป็นแบบไฮโดรไลซิส สิ่งที่น่าสนใจคือ การตัด pepsin เกิดขึ้นระหว่าง Leu 112-Tyr 113, Leu 166-Leu 167 และ Leu 178-Asn 179 ซึ่งการตัดเกิดขึ้นที่ปลายด้านคาร์บอกซิลของ leucine ทั้งสามตำแหน่ง ดังนั้น ในงานวิจัยนี้ผู้วิจัยจึงสังเคราะห์สารประกอบเชิงซ้อนของโมลิบดินัมชนิดใหม่ โดยใช้กรดอะมิโนเป็นลิแกนด์ของสารประกอบเชิงซ้อนโมลิบดินัม โดยในงานวิจัยนี้ได้เลือกใช้ leucine เป็นลิแกนด์ของสารประกอบเชิงซ้อนโมลิบดินัมและศึกษาการตัดโปรตีนตัวอย่าง คือ pepsin จากผลการทดลองพบว่า เกิดการตัดโปรตีนได้ 3 fragments ซึ่งเกิดจากการตัด pepsin 2 ตำแหน่ง และสามารถสรุปตำแหน่งที่ pepsin ถูกตัดได้ตำแหน่งเดียวคือระหว่าง Leu 112–Tyr 113 ปฏิกิริยาการตัด pepsin จะถูกยับยั้งโดย ethanol ดังนั้นผู้วิจัยคาดว่า hydroxyl radical น่าจะเป็น intermediate ที่เกิดขึ้นและมีผลต่อกลไกการตัดโปรตีนในปฏิกิริยานี้

ผลงานวิจัยนี้ได้ส่งไปยังวารสาร *J. Photochem. Photobiol. B* และกำลังรอผลตอบรับการตีพิมพ์

รายงานโครงการวิจัยฉบับสมบูรณ์

ชื่อโครงการ การออกแบบและสังเคราะห์โมเลกุลชนิดใหม่เพื่อศึกษาการจับและตัดโปรตีนที่ตำแหน่งจำเพาะ

Design and synthesis of novel reagent for specific binding and cleavage of proteins

1. Introduction

Protein cleaving reagents (chemical proteases) have been of recent interest in biological chemistry. Because of the extreme stability of the peptide bond (half life of ~7 years at room temperature, pH 7) [1], the development of such reagents is challenging. Reagents that can specifically cleave the protein backbone can be useful in chemistry and biology. Such reagents can be used to map ligand binding sites on proteins, DNA binding sites on proteins, and to explore protein-protein interactions. Protein sequencing studies, in addition, can utilize such reagents to produce smaller fragments that are more amenable for peptide sequencing. Successful design of new and efficient chemical peptidases also improves our understanding of the molecular basis for recognition of small molecules by proteins [2-8].

The design of such reagents should include, 1) appropriate recognition elements for binding to the target site with high affinity, and 2) chromophores which can be activated to produce the desired cleavage chemistry with high selectivity. Photocleavage of proteins by small, designed organic molecules has been developed in our laboratories [9-15]. Photoreactive chromophores linked to short peptides have been developed and protein photocleavage at the probe binding site was achieved. The resulting peptides are amenable to sequencing, an important feature for biochemical applications of these reagents. BSA, for example, was cleaved by *N*-[4(1-pyrene)butyroyl]-L-phenylalanine (Py-L-Phe) between Leu 346 and Arg 347, while lysozyme was cleaved between Trp 108 and Val 109. In the study, pyrenyl chromophore was selected in the modular design. The reason for choosing pyrenyl chromophore is due to its long-lived, high energy singlet excited state, which can be used to sensitize chemical reactions. Moreover, the pyrenyl chromophore has strong absorption bands in the near-UV region, which are sensitive to the environment of the chromophore, and strong fluorescence bands in the visible region, which are useful to monitor binding to proteins.

In this study, a new probe, d-biotinyl-1(1-pyrene) methylamide was synthesized by coupling d-biotin to 1(1-pyrene)methylamine hydrochloride using *N,N′*-dicyclohexyl carbodiimide (DCC). Current experiments are designed to test if Py-biotin would bind to avidin at the expected site on the protein and photocleave avidin at biotin binding site. Avidin is a tetrameric glycoprotein which binds biotin very tightly [16,17]. The protein is composed of four identical subunits with a molecular weight approximately 15 kDa per one subunit. The biotin-avidin interaction is one of the strongest noncovalent protein-ligand interactions found in nature [18]. The binding constant for this interaction is very high (10^{15} M^{-1}) and this feature prompted us to test avidin/biotin system for site-directed protein cleavage at biotin binding site on avidin.

The accessibility of the probe to the aqueous phase while binding to the protein can be estimated in fluorescence quenching studies. For the current studies, cobalt(III) hexammine trichloride ($Co(NH_3)_6Cl_3$, CoHA) was used as the fluorescence quencher since CoHA has only weak affinity for most proteins and CoHA is highly soluble in aqueous media. Moreover, CoHA quenches pyrenyl fluorescence at diffusion controlled rate constants. Quenching of the pyrenyl excited states by CoHA generates radical intermediates, which are capable of cleaving the peptide backbone. Therefore, the quenching constants with CoHA are expected to be useful indicators for the success of the photocleavage studies.

Another new pyrenyl probe, d-glucopyranosyl-1(1-pyrene) methylamide (Py-Glc) (Py-Glc) was synthesized in the same way. Py-Glc was used to test the photocleavage of sugar-binding proteins, such as lectin [19]. However, in the study we found that Py-Glc solution was not stable. Therefore, a new probe with a transition metal was designed.

Metal complexes have been also directed to specific cleavage of proteins by using affinity ligands. The achievement of peptide bond cleavage was accomplished *via* oxidative or hydrolytic methods [20-28]. The reactions can be activated using heat (thermal reaction) or light (photoreaction). However, the studies for protein photocleavage have not been widely investigated. These studies could be helpful in elucidating the role of metal ions in natural hydrolases. Photocleavage of lysozyme, for example, by Co(III) complexes has been reported [29]. Two photocleaved fragments were achieved upon irradiation of lysozyme-pentammineaquocobalt(III) complex at 310, 340, or 370 nm, at room temperature and pH 7.0. Searching for new molecules to expand this repertoire is very challenging. Molybdenum is one of the important metals found in metalloenzymes, and it is the only 4d

element with a biological function [30], and photocleavage of proteins by molybdenum complex has not been reported. Here, the ability of molybdenum complexes to selectively cleave a protein, under photochemical conditions, is investigated. A molybenum(VI) peroxo α-amino acid complex were synthesized by attaching an amino acid (leucine) to molybdenum trioxide (MoO_3). Porcine pepsin, a zymogen-derived protein, was chosen for the current study as a model protein since the complete amino-acid sequence of porcine pepsin and its crystal structure are known [31-32]. This new observation may provide the rational design of transition metal complexes for the footprinting of metal binding sites on proteins and in addition facilitate in the development of more efficient artificial proteases.

2. Materials and methods

2.1 Synthesis of the probes

2.1.1 Synthesis of Py-biotin

Py-biotin was synthesized by coupling 1(1-pyrene)methylamine hydrochloride (50.0 mg) with d-biotin (50.0 mg) by using *N,N′*-dicyclohexyl carbodiimide (0.10 g) The mixture was stirred at room temperature for 5 hours. Water (40.0 mL) was added to stop the reaction. Then the product was extracted with CH_2Cl_2 (3x10 mL), and purified using column chromatography (eluted with CH_2Cl_2/CH_3OH). The product (Py-biotin) was characterized by UV–VIS, fluorescence, ^{1}H NMR and mass spectroscopy.

2.1.2 Synthesis of Py-Glc

Py-Glc was synthesized by coupling 1(1-pyrene)methylamine hydrochloride (52.0 mg) with glucose (52.0 mg) by using *N,N′*-dicyclohexyl carbodiimide (79.0 mg) in pyridine (30 mL). The mixture was stirred at room temperature for 5 hours. Water (40.0 mL) was added to stop the reaction. Then the product was extracted with CH_2Cl_2 (3x10 mL), and purified using column chromatography (eluted with CH_2Cl_2/CH_3OH). The product (Py-Glc) was characterized by UV–VIS, fluorescence, ^{1}H NMR and mass spectroscopy.

2.1.3 Synthesis of $MoO(O_2)_2$(α-leucine) (H_2O)

$MoO(O_2)_2$(α-leucine) (H_2O) was synthesized by following the previously reported method [33]. MoO_3 (2.85 g) was dissolved in 30% H_2O_2 (10 mL) with stirring at 30°C for 24 hours. Leucine (2.70 g) was gradually added to the previous solution. The solution was stirred at 30°C for 24 h. The yellowish precipitate was obtained (5.51 g; 85.03% yield). The product was identified using UV-Vis, IR and ^{1}H NMR spectroscopy.

2.2 Binding studies

Binding of Py-biotin to avidin was investigated by spectroscopic techniques. Avidin solution (0-1.5 μM) was added to a solution of Py-biotin (4.2 μM) and the absorption spectra were recorded on a Shimadzu UV-2401 PC UV-VIS spectrophotometer. The fluorescence spectra were recorded on a Jasco FP-6200 spectrofluorometer. In fluorescence titration experiments, the pyrenyl probe was excited at 342 nm. The fluorescence intensity was monitored at 376 nm, as a function of protein concentration. In fluorescence quenching experiments, the probe-protein mixtures were titrated with CoHA and the fluorescence intensities have been monitored at 376 nm (excitation at 342 nm).

2.3 Photochemical Protein Cleavage

2.3.1 Cleavage of avidin by Py-biotin

The protein photocleavage was carried out in the dark room at room temperature. Avidin (15 μM), containing Py-biotin (15 μM) and CoHA (1 mM) (as an electron acceptor) in 50 mM Tris-HCl buffer, pH 7.0 (total volume 200 μL), was irradiated at 342 nm using a 150 W xenon lamp. Dark control samples were prepared under the same conditions, as described above, except that the solutions were protected from light. All reaction mixtures were lyophilized (freeze-drying) until dryness.

2.3.2 Cleavage of streptavidin by Py-biotin

The cleavage study of streptavidin was performed using the same procedure as described in the cleavage study of avidin. All reaction mixtures were lyophilized (freeze-drying) until dryness.

2.3.3 Cleavage of lysozyme by Py-Glc

The protein photocleavage was carried out in the dark room at room temperature. Lysozyme (15 μM), containing Py-Glc (15 μM) and CoHA (1 mM) (as an electron acceptor) in 50 mM Tris-HCl buffer, pH 7.0 (total volume 200 μL), was irradiated at 349 nm using a 150 W xenon lamp. Dark control samples were prepared under the same conditions, as described above, except that the solutions were protected from light. All reaction mixtures were dried under reduced pressure. The cleavage of other proteins, such as α-casein and lectin were also investigated using the same procedure.

2.3.4 Cleavage of pepsin by $MoO(O_2)_2$(α-leucine)(H_2O)

The protein cleavage was carried out at room temperature. Porcine pepsin (15 μM) was treated with $MoO(O_2)_2$(α-leucine)(H_2O) (2.0 mM) in 50 mM Tris-HCl buffer, pH 7.0 (total volume 200 μL), and the reaction mixtures were irradiated at 320 and 340 nm for 10-30 min with 150W xenon lamp. Dark control sample was prepared under the same conditions, as described above, except that the solution was protected from light. All reaction mixtures were lyophilized (freeze-drying) until dryness.

2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE Experiments were performed following literature methods with minor modifications [34]. The dried sample residue was redissolved in sample loading buffer (glycerol (1.2 mL), sodium dodecyl sulfate (7% w/v), Tris–HCl (50 mM, pH 6.8),

bromophenol blue (0.01% w/v) and 2-mercaptoethanol (2%, v/v)). Protein samples in loading buffer were heated for 3 minutes before loading onto the gel. The gels (10-12%) were run by applying 60 V until the dye passed through the stacking gel. The voltage was then increased to 110 V. The gels were run for 1.5 h, stained with Coomassie brilliant blue, and destained in acetic acid solution (10%).

2.5 Peptide transfer and amino acid sequencing

The separated peptide fragments on SDS-polyacrylamide gel were transferred to PVDF membrane with a current of 140 mA for 1 h using the semi-dry system (BIORAD) with CAPS buffer, pH 10.5. The transferred protein fragments on PVDF membrane were stained with Coomassie brilliant blue (0.1% Coomassie brilliant blue R-250 in 40% methanol and 1% acetic acid). The desired bands were cut and sent for N-terminal amino acid composition analysis (Midwest Analytical, Inc., MO, USA). Chemical sequencing was performed on an automated protein sequencer. Five cycles were performed to identify the N-terminus of the cleaved fragments.

2.6 Inhibition study

2.6.1 Inhibition of avidin cleavage

In the inhibition study, the photoreactions were performed as above but d-biotin (60 μM) was mixed with the protein solution for 15 minutes prior to addition of Py-biotin (15 μM) and CoHA (1 mM). The solutions were then irradiated at 342 nm as described above.

2.6.2 Inhibition of pepsin cleavage

The participation of hydroxyl radical intermediate in the cleavage reaction is tested in quenching studies with ethanol. Ethanol (0.5 mM) was added to the reaction mixture (pepsin + $MoO(O_2)_2$(α-leucine)(H_2O)), and the reaction mixture was irradiated at 340 nm for 20 minutes.

3. Results

3.1 Synthesis of the probes

3.1.1 Synthesis of Py-biotin

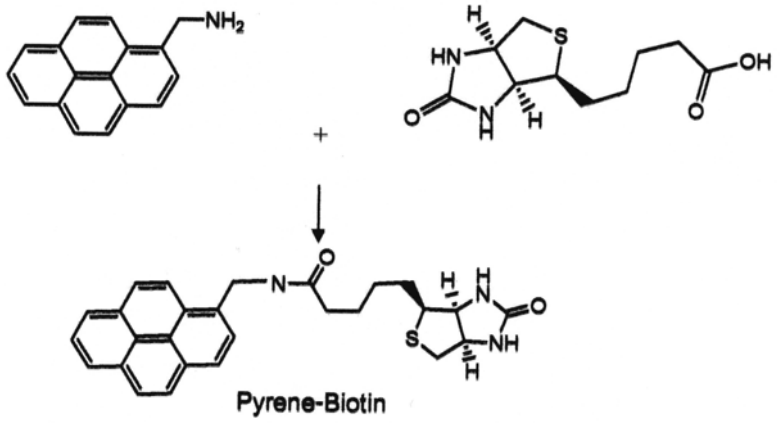


Fig. 1 Structure of d-biotinyl-1(1-pyrene)methylamide (Py-biotin).

Spectroscopic data:

UV-Vis absorption spectrum: λ_{max} = 342 nm

^{1}H NMR (400 MHz, 10%MeOD/$CDCl_3$): δ7.8–8.2 (9H), 5.0 (1H), 4.1 (1H), 3.8 (1H), 3.3 (2H), 2.5-2.6 (2H), 2.2 (2H), 1.1–1.7 (6H)

Mass spectral data: *m/z* 456.4 (M^+)

3.1.2 Synthesis of Py-Glc

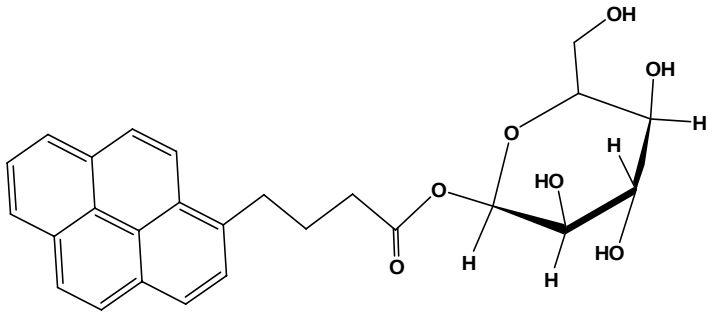


Fig. 2 Structure of d-glucopyranosyl-1(1-pyrene) methylamide (Py-Glc).

Spectroscopic data:

UV-Vis absorption spectrum: λ_{max} = 349 nm

^{1}H NMR (400 MHz, $CDCl_3$): δ7.9–8.4 (9H), 5.5-5.6 (1H), 3.9 (2H), 3.3-3.5 (5H), 2.5 (2H), 2.2 (2H), 1.9-2.1 (2H)

Mass spectral data: *m/z* 450.1 (M^+)

3.1.3 Synthesis of $MoO(O_2)_2$(α-leucine)(H_2O)

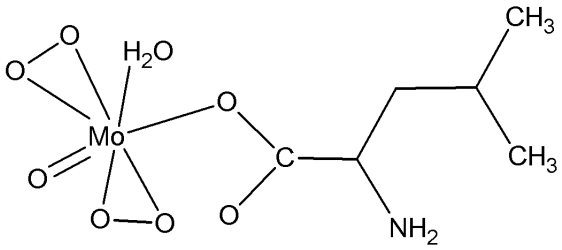


Fig. 3 Structure of molybenum(VI) peroxo α-amino acid complexes ($MoO(O_2)_2$(α-leucine)(H_2O)).

Spectroscopic data:

UV-Vis absorption spectrum: λ_{max} = 250 nm

IR spectrum : M=O 980.26 cm^{-1}, O-O 847.59 cm^{-1}, M-$O(O)_2$ 630.16 cm^{-1}, M-$O(O)_2$ 531.35 cm^{-1}

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.82 ppm (1H), 2.67 ppm (2H), 1.98 ppm (1H),0.98 ppm (6H)

3.2 Binding studies using spectroscopic techniques

Binding of Py-biotin to avidin resulted in red shifts of the absorption peak positions of the pyrenyl chromophore with an isosbestic point at 342.5 nm followed by hyperchromism (Fig. 4) with the binding constant of 8.0 x 10^6 dm^3 mol^{-1} and binding site of 4. The data indicate a multimodal binding with several binding states, which is consistent with the fact that there are at least 4 distinct sites where biotin is known to bind. The red shifts in the absorption spectra with increase in protein concentration followed by hyperchromism clearly indicate that the pyrenyl chromophore sensing environmental changes upon binding to the protein.

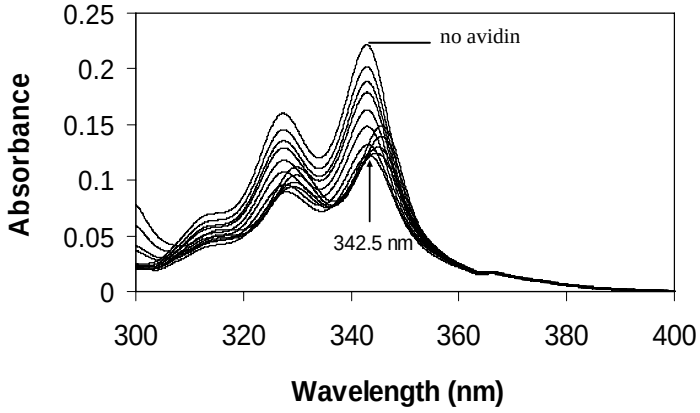


Fig. 4 Absorption spectra of Py-biotin with increasing concentrations of avidin. The initial decrease in the absorbance with the red shift (342 to 345 nm) of the peak positions and the isosbestic point at 342.5 nm are evident in the spectra.

The fluorescence of Py-biotin (2.0 μM) was enhanced by the gradual addition of avidin (0-10 μM, excitation at 342 nm). These observations indicate that the pyrenyl chromophore is interacting with the protein matrix to form protein/Py-biotin complex. No shifts in peak positions and no new bands of pyrene excimer emission were observed. Enhanced fluorescence intensities of Py-biotin upon binding to avidin can be expected. These results may be due to the protection of the probe from solvent, oxygen, and other quenchers. These aspects are further examined in fluorescence quenching experiments which will provide more information of the probe accessibility to CoHA in aqueous solution.

The accessibility of the pyrenyl chromophore of Py-biotin to the solvent was probed in the fluorescence quenching studies, with CoHA. The emission from the avidin-bound Py-biotin was quenched marginally by the addition of CoHA while in the absense of the protein, the probe emission has been quenched very strongly (Fig. 5). Quenching constant (K_{sv}) was obtained from Stern-Volmer equation [35]:

$$I_0/I = 1 + K_{sv}\,[\text{CoHA}] \qquad (1)$$

Where I_0 is the fluorescence intensity in the absence of the quencher, and I is the intensity in the presence of the quencher. The plot of I_0/I as a function of CoHA concentration (Eq. 1) gives the K_{sv} value.

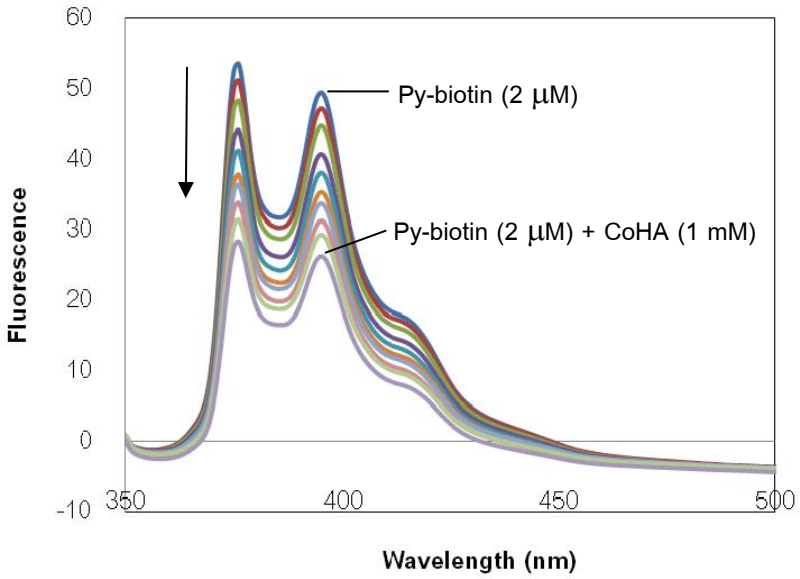


Fig. 5 Fluorescence spectra of Py-biotin (2 μM) with increasing concentrations of CoHA (0-1.0 mM). Excitation wavelength is 342 nm.

Analysis of the data by the Stern-Volmer equation indicated that K_{sv} value of 1378.7 M^{-1} (no protein) decreased dramatically when Py-biotin was bound to avidin (10 μM) with the quenching slope reduced to ~170 M^{-1} (Fig. 6). Fluorescence quenching data, therefore, indicates the protection of Py-biotin fluorescence by the protein matrix.

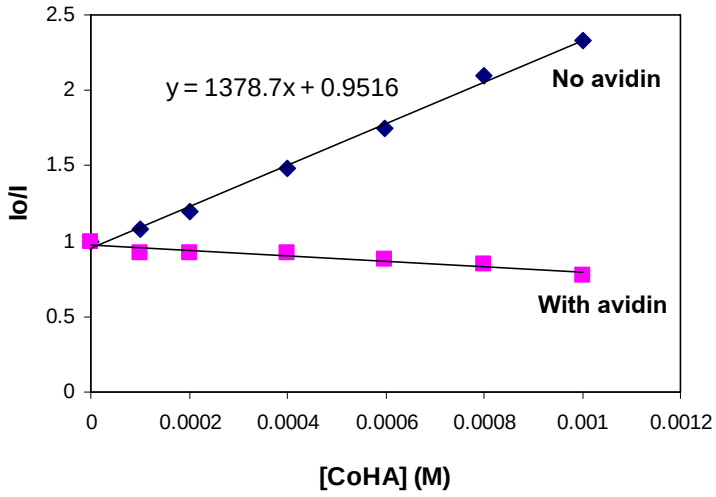


Fig. 6 Quenching plots of the Py-biotin (2 μM) by CoHA, in buffer (◆), and in the presence of avidin (10 μM, (■)).

3.3 Photocleavage studies

3.3.1 Photocleavage of avidin by Py-biotin

Avidin was successfully cleaved by Py-biotin. The protein photocleavage was monitored in gel electrophoresis experiments under denaturing conditions. Irradiation of avidin (15 μM)/Py-biotin (15 μM) complex, in the presence of CoHA (1 mM), at 342 nm resulted in efficient cleavage of the protein backbone at specific site, as demonstrated by SDS-PAGE (Fig. 7). Two new bands were observed with molecular weights of ~9 and ~5 kDa (lanes 8-9) and the sum of the molecular masses of the fragments roughly add-up to that of the intact avidin. The photoreaction requires the probe, an electron acceptor (lane 4, no CoHA) and light (lane 3). No cleavage was observed when avidin was irradiated in the absence of either the two reagents or light in the control lanes. Yields of photoproducts increase steadily with irradiation time.

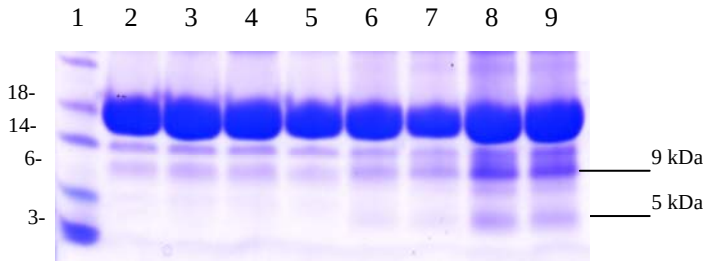


Fig. 7 Site specific photocleavage of avidin by Py-biotin. Lane 1 contained molecular weight markers as indicated (kDa). Lane 2 contained avidin (15 μM). Lanes 3-4 contained avidin (15 μM) and Py-biotin (15 μM). Lanes 5-9 contained avidin (15 μM), Py-biotin (15 μM) and CoHA (1 mM). (Lanes 3, 5 were the dark controls while samples in lanes 4, 6-9 were exposed to 342 nm radiation for 20, 5, 10, 15 and 20 minutes, respectively.)

3.3.2 Photocleavage of streptavidin by Py-biotin

Irradiation of streptavidin (15 μM)/Py-biotin (15 μM) complex, in the presence of CoHA (1 mM), at 342 nm did not result in cleavage of the protein backbone, as demonstrated by SDS-PAGE (Fig. 8).

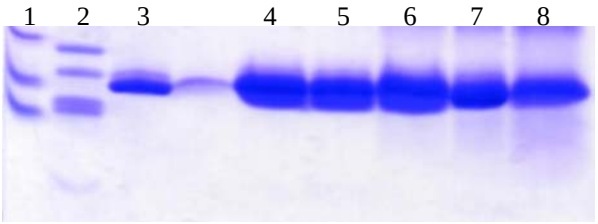


Fig. 8 Site specific photocleavage of streptavidin by Py-biotin. Lanes 1-2 contained molecular weight markers. Lane 3 contained streptavidin (15 μM). Lane 4 contained streptavidin (15 μM) and CoHA (1 mM). Lanes 5-8 contained streptavidin (15 μM), Py-biotin (15 μM) and CoHA (1 mM). (Lane 5 was the dark control while samples in lanes 4, 6-8 were exposed to 342 nm radiation for 20, 5, 10 and 20 minutes, respectively.)

3.3.3 Photocleavage of proteins by Py-Glc

Photocleavage of lysozyme

Irradiation of lysozyme (15 μM)/Py-Glc (15 μM) complex, in the presence of CoHA (1 mM), at 349 nm resulted in cleavage of the protein backbone, as demonstrated by SDS-PAGE (Fig. 9) Two new bands were observed with molecular weights of ~10 and ~4 kDa (lanes 6-7).

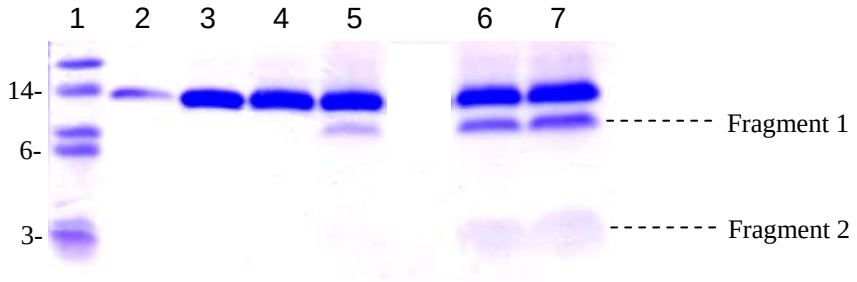


Fig. 9 Site specific photocleavage of lysozyme by Py-Glc. Lane 1 contained molecular weight markers as indicated (kDa). Lane 2 contained lysozyme (15 μM). Lane 3 contained lysozyme (15 μM) and Py-Glc (15 μM). Lanes 4-7 contained lysozyme (15 μM), Py-Glc (15 μM) and CoHA (1 mM). (Lane 4 was the dark control while samples in lanes 3, 5-7 were exposed to 349 nm radiation for 20, 5, 15 and 20 minutes, respectively.)

Photocleavage of α-casein

Irradiation of α-casein (15 μM)/Py-Glc (15 μM) complex, in the presence of CoHA (1 mM), at 349 nm did not result in cleavage of the protein backbone, as demonstrated by SDS-PAGE (Fig. 10)

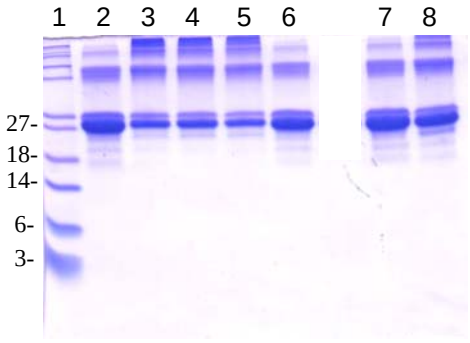


Fig. 10 Site specific photocleavage of α-casein by Py-Glc. Lane 1 contained molecular weight markers as indicated (kDa). Lanes 2-5 contained α-casein (15 μM), Py-Glc (15 μM) and CoHA (1 mM). Lane 6 contained α-casein (15 μM) and Py-Glc (15 μM). Lanes 7-8 contained α-casein (15 μM) and CoHA (1 mM). (Lanes 2 and 7 were the dark controls while samples in lanes 3-5 were exposed to 349 nm radiation for 5, 10, and 20 minutes, respectively. Samples in lanes 6 and 8 were exposed to 349 nm radiation for 20 minutes.)

Photocleavage of lectin

Irradiation of lectin (15 μM)/Py-Glc (15 μM) complex, in the presence of CoHA (1 mM), at 349 nm did not result in cleavage of the protein backbone, as demonstrated by SDS-PAGE (Fig. 11)

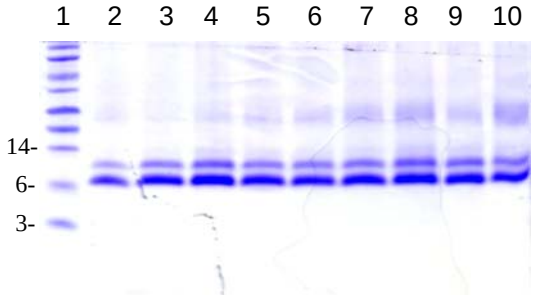


Fig. 11 Site specific photocleavage of lectin by Py-Glc. Lane 1 contained molecular weight markers as indicated (kDa). Lane 2 contained lectin (15 μM). Lanes 3-10 contained

lectin (15 μM), Py-Glc (15 μM) and CoHA (1 mM). (Lane 3 was the dark control while samples in lanes 4-10 were exposed to 349 nm radiation for 5, 10, 15, 20, 30, 40 and 60 minutes, respectively.)

3.3.4 Photocleavage of pepsin by $MoO(O_2)_2$(α-leucine) (H_2O)

Pepsin was successfully cleaved by molybdenum complex, under activation by light, at mild conditions (room temperature, pH 7.0). Irradiation of pepsin (15 μM) in the presence of $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM) at 320 nm and 340 nm for up to 30 minutes resulted in cleavage of the protein as demonstrated in Fig. 12. The cleaved pepsin resulted in at least three fragments (I, II and III) with the molecular weights of ~25, ~20 and ~12 kDa, respectively (lanes 4-8). However, irradiation of the protein-$MoO(O_2)_2$(α-leucine) (H_2O) mixture at 320 nm for 10-30 minutes gave faint bands of the cleaved fragments (lanes 3-5, respectively), compared to the results obtained at 340 nm (lanes 6-8). No cleavage was observed in the absence of the light (dark control, lane 2) and $MoO(O_2)_2$(α-leucine) (H_2O) (data not shown).

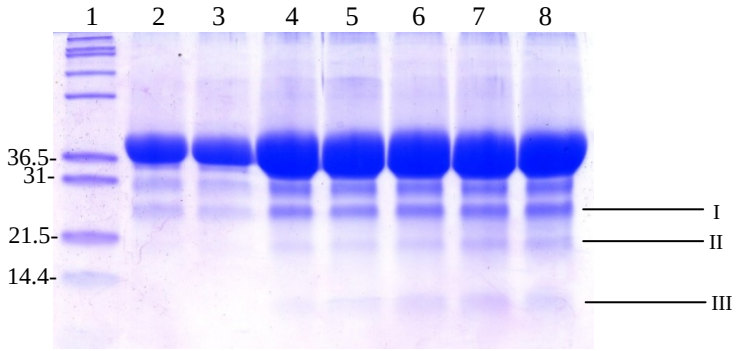


Fig. 12 SDS-PAGE of the photocleaved products of pepsin by $MoO(O_2)_2$(α-leucine) (H_2O). Lane 1 contained molecular weight markers as indicated (kDa). Lanes 2-8 contained pepsin (15 μM) and $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM). Lane 2 was the dark control. Samples in lanes 3-5 were irradiated at 320 nm for 10, 20, and 30 min, respectively while samples in lanes 6-8 were irradiated at 340 nm for 10, 20, and 30 min, respectively.

The concentration of $MoO(O_2)_2$(α-leucine)(H_2O) in pepsin cleavage study was varied (0.125-2.0 mM). The results showed the increase of cleavage yield when increasing $MoO(O_2)_2$(α-leucine)(H_2O) concentration, as shown in Fig. 13.

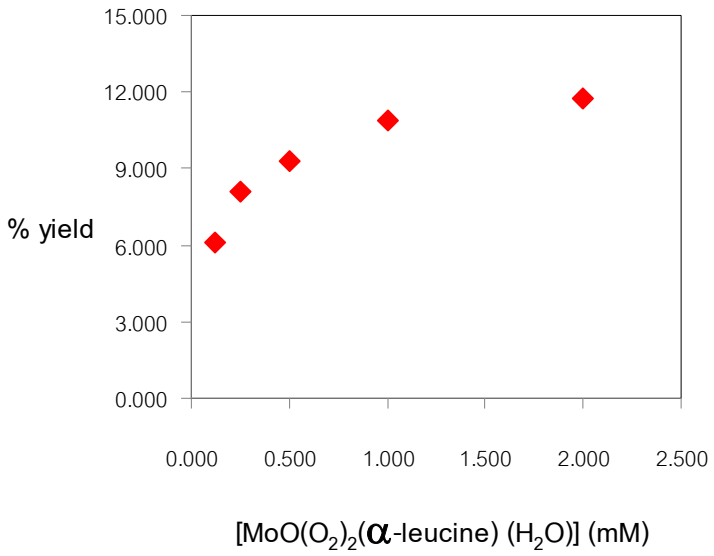


Fig. 13 Plot of %yield of the cleaved fragments vs [$MoO(O_2)_2$(α-leucine) (H_2O)].

3.4 Amino acid sequencing and cleavage sites determination

3.4.1 Amino acid sequencing of avidin cleavage

To locate the cleavage site on avidin, the peptide fragments from the gels were isolated and subjected to amino acid sequencing. The newly generated N-terminal sequences of the cleaved fragments were successfully sequenced. N-terminal sequencing of both fragments (~9 kDa and ~5 kDa) indicated the residues ARKCS, which is the known N-terminal sequence of avidin [36-37]. However, a minor product with the N-terminal sequence of VFTGQ, a sequence internal to avidin, was also observed with the ~5 kDa fragment. From the known sequence of avidin, we deduce that the cleavage occurs between Thr 77 and Val 78.

77 ↓ 78

H_2N-Ala-Arg-Lys-Cys……....Arg-Thr – Val-Phe-Thr-Gly……..……COOH

N-terminal fragment (~9 kDa) C-terminal fragment (~5 kDa)

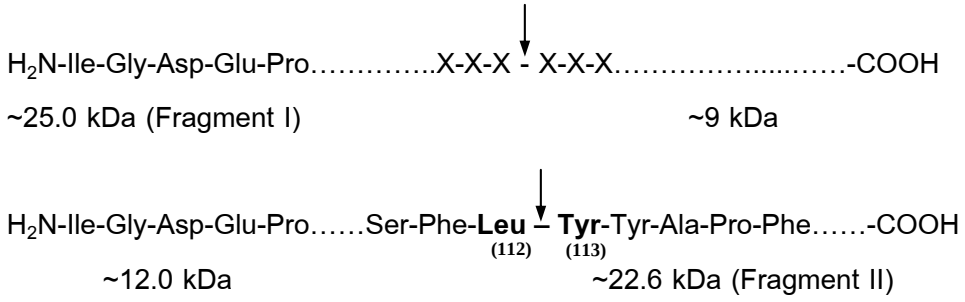
The crystal structure of avidin shows that this region is proximate to the biotin binding site in the 3D structure of avidin [38]. The structure of the binding site residues is highly complementary to that of biotin molecule, accounting for specific recognition. If the biotinyl part of Py-biotin binds at the biotin binding site with the pyrenyl chromophore buried at the hydrophobic cavity in the protein near the binding site, then the cleavage could result in the observed region.

3.4.2 Amino acid sequencing of lysozyme cleavage

Since Py-Glc used in the reaction was not quite stable. Therefore, The amino acid sequence of the cleaved fragments was not analyzed.

3.4.3 Amino acid sequencing of pepsin cleavage

To locate the cleavage sites on pepsin, the peptide fragments from the gels were isolated and subjected to amino acid sequencing. N-terminal sequencing of fragment I (~25 kDa) indicated the sequence IGDEP which corresponds to the known N-terminal sequence of native pepsin [39-40]. N-terminal sequencing of fragment II (~20 kDa) showed an amino acid sequence YYAPF. The sequence of YYAPF indicated the cleavage site at Leu 112 -Tyr 113. The protein band of fragment III (~12 kDa) was very faint, and the observed N-terminal sequencing data were not clear. This might be due to the insufficient amount of the blotted protein for the sequence analysis, or the fragment may not be amenable to sequencing. Therefore, we can conclude that three observed cleaved fragments may arise from two cleavage sites, with a small amount of another fragment (~9 kDa) that is not distinguishable in the gels. However, only one cleavage site was clearly concluded to be between Leu 112 -Tyr 113.



From the data above, the cleavage between Leu 112 -Tyr 113 gives another sequence (N-terminal sequence) with molecular weight of ~12 kDa, which could possibly be the observed fragment III on the gels.

3.5 Inhibition study

3.5.1 Inhibition of avidin cleavage

In the inhibition study, d-biotin (60 μM) was mixed with the protein solution for 15 minutes prior to addition of Py-biotin (15 μM) and CoHA (1 mM). The solutions were then irradiated at 342 nm as described above. Irradiation of the above mixture solution at 342 nm resulted to the decrease of photocleavage yields (Fig. 14). The inhibition study is consistent with the cleavage site obtained from the amino acid sequencing. d-Biotin may occupy the biotin binding sites on avidin. Therefore, the specific binding site is not available for the incoming Py-biotin.

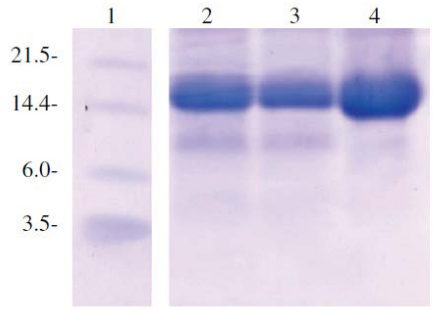


Fig. 14 Inhibition of photocleavage of avidin by d-biotin. Lane 1 contained molecular weight markers as indicated (kDa). Lanes 2-3 contained avidin (15 μM), Py-biotin (15 μM) and CoHA (1 mM), exposed to 342 nm radiation for 15 and 10 minutes, respectively. Lane 4 contained avidin/biotin mixture (15 μM), Py-biotin (15 μM) and CoHA (1 mM), exposed to 342 nm radiation for 15 minutes.

3.5.2 Quenching of pepsin cleavage reaction by ethanol

Exposure of peptides to hydroxyl radical is known to effect backbone cleavage. The participation of hydroxyl radical intermediate in the cleavage is tested in quenching studies with ethanol. Ethanol (0.5 mM) was added to the pepsin/ $MoO(O_2)_2$(α-

leucine) (H_2O) mixture, and the mixture was irradiated at 340 nm for 20 minutes. The cleavage of pepsin is quenched by ethanol, as shown in Fig. 15

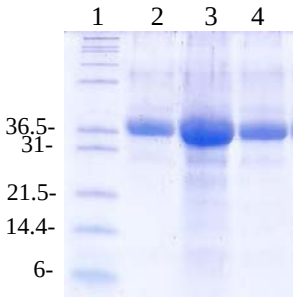


Fig. 15 Quenching of photocleavage of pepsin by ethanol. Lane 1 contained molecular weight markers as indicated (kDa). Lanes 2-3 contained pepsin (15 μM) and $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM). Lane 4 contained pepsin (15 μM), $MoO(O_2)_2$(α-leucine) (H_2O) (2.0 mM) and ethanol (0.5 mM). Lane 2 was the dark control, while samples in lanes 3-4 were irradiated at 340 nm for 20 min.

4. Conclusion

Absorption and fluorescence spectral data clearly indicate the binding of the pyrenyl probe (Py-biotin) to avidin. Fluorescence quenching studies suggest that Py-biotin binding site in avidin is buried in the protein matrix, away from the aqueous phase. Pyrenyl chromophore linked to biotin can be delivered to the specific site on avidin. Upon photoexcitation of pyrene, in the presence of an electron acceptor, the cleavage of avidin can occur in the proximity to the biotin binding site. The formation of pyrenyl cation radical resulted from quenching of pyrene excited state by CoHA is expected to play the important role in the photoreaction. The cleavage efficiency of the probe correlates with the accessibility of the protein-bound probe to CoHA. Thus, the accessibility of the pyrenyl chromophore controls efficiency. To improve the accessibility of the protein-bound probe to the quencher, conjugation of a quencher to the probe molecule could be a possible way to enhance the cleavage efficiency in the future studies.

The data in the current studies clearly show that $MoO(O_2)_2$(α-leucine) (H_2O) successfully cleaves pepsin at specific sites under photochemical conditions. The cleavage can be achieved when the reaction mixture is irradiated for only 10 minutes. Cleavage

specificity may occur due to specific binding of the metal complex to the selective sites on the protein. From the above results, hydroxyl radicals may be responsible for the cleavage of the protein, as indicated by the decrease of cleavage yields in the presence of ethanol. Hydroxyl radicals may be generated at or near the cleavage sites. However, the mechanism for photocleavage of pepsin needs more investigation. Even though the yields are small (<15%), but the photocleavage reaction has shown high selectivity. This study will be useful for the footprinting of metal binding sites on specific proteins in the future.

In conclusions, the data in the current studies clearly show that the protein cleavage site can be controlled by appending a desired ligand to a chromophore, thus paving the way for a rational approach to target particular sites on proteins. Therefore, this will be helpful in designing site-specific reagents for the photocleavage of proteins. In addition to biochemical applications, such reagents may be of interest for therapeutic purposes.

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Outputs ที่ได้จากงานวิจัย

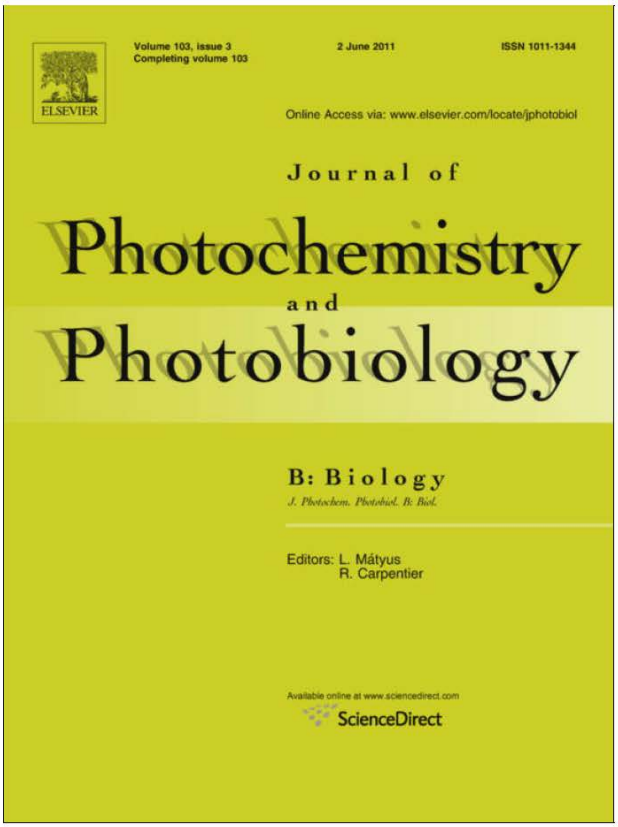
มีผลงานวิจัยที่ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 1 เรื่องคือ

Malaikaew, P.; Svasti, J.; Kumar, C. V.; *Buranaprapuk, A.* (2011) Photocleavage of avidin by a new pyrenyl probe. *J. Photochem. Photobiol. B*, 103, 251–255. (impact factor = 2.077)

และกำลังรอผลตอบรับการตีพิมพ์จำนวน 1 เรื่องคือ

Jityuti, B.; Liwporncharoenvong, T.; *Buranaprapuk, A.* (2013) Use of A Molybdenum(VI) Complex as Artificial Protease in Protein Photocleavage. *J. Photochem. Photobiol. B*, submitted for publication.

ภาคผนวก



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Photocleavage of avidin by a new pyrenyl probe

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Biotin

ABSTRACT

In this study, a new small-molecule-based reagent was designed to recognize and bind to specific site in protein. A new pyrenyl probe, d-biotinyl-1(1-pyrene)methylamide (Py–biotin) was designed and synthesized by coupling of d-biotin to 1(1-pyrene)methylamine hydrochloride. Binding studies and site-specific photocleavage of avidin by Py–biotin were demonstrated. Binding of Py–biotin to avidin was studied using absorbance and fluorescence spectroscopic techniques. Red shifts of the absorption peak positions of the pyrenyl chromophore followed by hyperchromism were observed upon binding to avidin. The photocleavage of avidin was achieved when a mixture of the protein, Py–biotin, and an electron acceptor, cobalt(III) hexammine trichloride (CoHA), was irradiated at 342 nm. No reaction occurred in the absence of the probe, CoHA, or light. *N*-terminal sequencing of the peptide fragments indicated a cleavage site of avidin between Thr 77 and Val 78. The high specificity of photocleavage may be valuable in targeting specific sites of proteins with small molecules.

1. Introduction

Protein cleaving reagents (chemical proteases) have been of recent interest in biological chemistry. Because of the extreme stability of the peptide bond (half life of ~7 years at room temperature, pH 7) [1], the development of such reagents is challenging. Reagents that can specifically cleave the protein backbone can be useful in chemistry and biology [2–6]. Such reagents can be used to map ligand binding sites on proteins, DNA binding sites on proteins, and to explore protein–protein interactions. Protein sequencing studies, in addition, can utilize such reagents to produce smaller fragments that are more amenable for peptide sequencing. Successful design of new and efficient chemical peptidases also improve our understanding of the molecular basis for recognition of small molecules by proteins [7–11].

The design of such reagents should include, (1) appropriate recognition elements for binding to the target site with high affinity, and (2) chromophores which can be activated to produce the desired cleavage chemistry with high selectivity. Photocleavage of proteins by small, designed organic molecules is being developed in our laboratories [12,13]. Photoreactive chromophores linked to short peptides have been developed and protein photocleavage at the probe binding site was achieved. The resulting peptides are amenable to sequencing, an important feature for biochemical applications of these reagents. BSA, for example, was cleaved by *N*-[4(1-pyrene)butyroyl]-L-phenylalanine (Py-L-Phe) between Leu 346 and Arg 347, while lysozyme was cleaved between Trp 108 and Val 109. In the study, pyrenyl chromophore was selected in the modular design. The reason for choosing pyrenyl chromophore is due to its long-lived, high energy singlet excited state, which can be used to sensitize chemical reactions [14]. Moreover, the pyrenyl chromophore has strong absorption bands in the near-UV region, which are sensitive to the environment of the chromophore, and strong fluorescence bands in the visible region, which are useful to monitor binding to proteins [15,16].

Avidin is a tetrameric glycoprotein which binds biotin very tightly [17,18]. The protein is composed of four identical subunits with a molecular weight approximately 15 kDa per one subunit. The biotin–avidin interaction is one of the strongest noncovalent protein–ligand interactions found in nature [19]. The binding constant for this interaction is very high ($10^{15}\ M^{-1}$) and this feature prompted us to test avidin/biotin system for site-directed protein cleavage at biotin binding site on avidin. A new probe, d-biotinyl-1(1-pyrene)methylamide was synthesized by coupling d-biotin to 1(1-pyrene)methylamine hydrochloride using *N*,*N*′-dicyclohexyl carbodiimide (DCC) by suitable modification of reported method (Scheme 1) [20]. Current experiments are designed to test if Py–biotin would bind to avidin at the expected site on the protein and photocleave avidin at biotin binding site. Binding studies of Py–biotin to avidin was investigated by absorption and fluorescence spectroscopy.

The accessibility of the probe to the aqueous phase while binding to the protein can be estimated in fluorescence quenching

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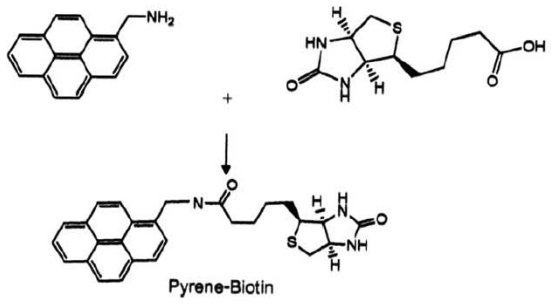

Scheme 1. Structure of d-biotinyl-1(1-pyrene)methylamide (Py–biotin), synthesized by coupling of d-biotin to 1(1-pyrene)methylamine hydrochloride.

studies. For the current studies, cobalt(III) hexammine trichloride ($Co(NH_3)_6Cl_3$, CoHA) was used as the fluorescence quencher since CoHA has only weak affinity for most proteins and CoHA is highly soluble in aqueous media. Moreover, CoHA quenches pyrenyl fluorescence at diffusion controlled rate constants. In the previous studies, CoHA has been used as an electron acceptor to induce protein cleavage with pyrenyl probes [13]. Quenching of the pyrenyl excited states by CoHA generates radical intermediates, which are capable of cleaving the peptide backbone. Therefore, the quenching constants with CoHA are expected to be useful indicators for the success of the photocleavage studies.

2. Materials and methods

Avidin (Mol. Wt. = 66,000) and d-biotin were purchased from Sigma Chemical Co. 1(1-pyrene)methylamine hydrochloride and *N,N′*-dicyclohexyl carbodiimide (DCC) were obtained from Aldrich. All solutions were freshly prepared in deionized water with 50 mM Tris–HCl buffer, pH 7.0.

2.1. Synthesis of Py–biotin

Py–biotin was synthesized by coupling 1(1-pyrene)methylamine hydrochloride (50.0 mg) with d-biotin (50.0 mg) by using *N,N′*-dicyclohexyl carbodiimide (0.10 g), as described in the previous report [20]. The product (Py–biotin) was characterized by UV–VIS, fluorescence, ^{1}H NMR and mass spectroscopy. ^{1}H NMR (400 MHz; d_6-DMSO): 7.8–8.2 (9H), 5.0 (1H), 4.1 (1H), 3.8 (1H), 3.3 (2H), 2.5–2.6 (2H), 2.2 (2H), 1.1–1.7 (6H); MS data: *m/z* 456.4 (M^+).

2.2. Spectroscopic studies

The absorption spectra were recorded on a Shimadzu UV-2401 PC UV–VIS spectrophotometer. The fluorescence spectra were recorded on a Jasco FP-6200 spectrofluorometer. In fluorescence titration experiments, the pyrenyl probe was excited at 342 nm. The fluorescence intensity was monitored at 376 nm, as a function of protein concentration. In fluorescence quenching experiments, the probe–protein mixtures were titrated with CoHA and the fluorescence intensities have been monitored at 376 nm (excitation at 342 nm).

2.3. Photochemical protein cleavage

The protein photocleavage was carried out in the dark room at room temperature. The protein solution (15 μM), containing Py–biotin (15 μM) and CoHA (1 mM) in 50 mM Tris–HCl buffer, pH 7.0 (total volume 200 μL), was irradiated at 342 nm using a 150 W xenon lamp. A UV cutoff filter (WG-345; 78%T at 342 nm) was used to remove stray UV light. Dark control samples were prepared under the same conditions, as described above, except that the solutions were protected from light. All reaction mixtures were dried under reduced pressure.

In the inhibition study, the photoreactions were performed as above but d-biotin (60 μM) was mixed with the protein solution for 15 min prior to addition of Py–biotin (15 μM) and CoHA (1 mM). The solutions were then irradiated at 342 nm as described above.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE Experiments were performed following literature methods with minor modifications [21]. The dried sample residue was redissolved in sample loading buffer (glycerol (1.2 ml), sodium dodecyl sulfate (7%, w/v), Tris–HCl (50 mM, pH 6.8), bromophenol blue (0.01%, w/v) and 2-mercaptoethanol (2%, v/v)). Protein samples in loading buffer were heated for 3 min before loading onto the gel. The gels (10%) were run by applying 60 V until the dye passed through the stacking gel. The voltage was then increased to 110 V, as described in the previous report [22]. The gels were run for 1.5 h, stained with Coomassie brilliant blue, and destained in acetic acid solution (10%).

2.5. Peptide transfer and amino acid sequencing

The separated peptide fragments on SDS–polyacrylamide gel were transferred to PVDF membrane with a current of 60 mA for 1.5 h using the semi-dry system (BIORAD) with CAPS buffer, pH 10.5. The transferred protein fragments on PVDF membrane were stained with Coomassie brilliant blue (0.1% Coomassie brilliant blue R-250 in 40% methanol and 1% acetic acid). The desired bands were cut and sent for *N*-terminal amino acid composition analysis (Midwest Analytical, Inc., MO, USA). Chemical sequencing was performed on an automated protein sequencer. Five cycles were performed to identify the *N*-terminus of the cleaved fragments.

3. Results and discussion

3.1. Binding studies using spectroscopic techniques

Binding of Py–biotin to avidin was investigated by spectroscopic techniques. Addition of avidin solution (0–1.5 μM) to a solution of Py–biotin (4.2 μM) resulted in red shifts of the absorption peak positions of the pyrenyl chromophore with an isosbestic point at 342.5 nm followed by hyperchromism (Fig. 1). The data

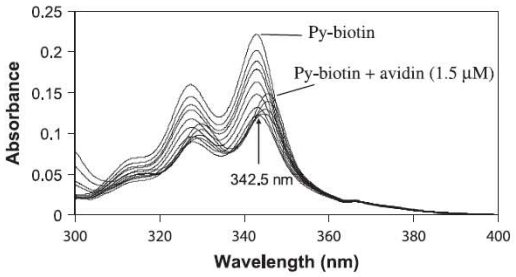

Fig. 1. Absorption spectra of Py–biotin (4.2 μM) (1 cm path length) with increasing concentrations of avidin (0–1.5 μM). The initial decrease in the absorbance with the red shift (342–345 nm) of the peak positions and the isosbestic point at 342.5 nm are evident in the spectra.

indicate a multimodal binding with several binding states, which is consistent with the fact that there are at least four distinct sites, where biotin is known to bind. Therefore, multiple binding events are being resolved to some degree, in the spectra. The red shifts in the absorption spectra with increase in protein concentration followed by hyperchromism clearly indicate that the pyrenyl chromophore sensing environmental changes upon binding to the protein.

Binding of Py–biotin to avidin was also confirmed by fluorescence studies. The fluorescence of Py–biotin (2.0 μM) was enhanced by the gradual addition of avidin (0–10 μM, excitation at 342 nm) (data not shown). These observations indicate that the pyrenyl chromophore is interacting with the protein matrix to form protein/Py–biotin complex. No shifts in peak positions and no new bands of pyrene excimer emission were observed. Enhanced fluorescence intensities of Py–biotin upon binding to avidin can be expected. These results may be due to the protection of the probe from solvent, oxygen, and other quenchers [15]. These aspects are further examined in fluorescence quenching experiments which will provide more information of the probe accessibility to CoHA in aqueous solution.

The accessibility of the pyrenyl chromophore of Py–biotin to the solvent was probed in the fluorescence quenching studies, with CoHA. The emission from the avidin-bound Py–biotin was quenched marginally by the addition of CoHA while in the absense of the protein, the probe emission has been quenched very strongly (Fig. 2). Quenching constant (K_{sv}) was obtained from Stern–Volmer equation [23]:

$$I_0/I = 1 + K_{sv}[CoHA] \quad (1)$$

where I_0 is the fluorescence intensity in the absence of the quencher, and I is the intensity in the presence of the quencher. The plot of I_0/I as a function of CoHA concentration (Eq. (1)) gives the K_{sv} value. Analysis of the data by the Stern–Volmer equation indicated that K_{sv} value of 1400 M^{-1} (no protein) decreased dramatically when Py–biotin was bound to avidin (10 μM) with the quenching slope reduced to ~170 M^{-1} (Fig. 3). Fluorescence quenching data, therefore, indicates the protection of Py–biotin fluorescence by the protein matrix.

3.2. Photocleavage of avidin

Avidin was successfully cleaved by Py–biotin. The protein photocleavage was monitored in gel electrophoresis experiments under denaturing conditions. Irradiation of avidin (15 μM)/Py–biotin (15 μM) complex, in the presence of CoHA (1 mM), at 342 nm resulted in efficient cleavage of the protein backbone at specific site, as demonstrated by SDS–PAGE (Fig. 4). Two new bands were observed with molecular weights of ~9 and ~5 kDa (lanes 4–7) and the sum of the molecular masses of the fragments roughly add-up to that of the intact avidin. The photoreaction requires the probe (lane 9, no Py–biotin), an electron acceptor (lane 8, no CoHA) and light (lane 3). No cleavage was observed when avidin was irradiated in the absence of either the two reagents or light in the control lanes. Yields of photoproducts increase steadily with irradiation time. However, the second product band (~5 kDa) in lanes 4 and 5 is too faint to see in Fig. 4. Photocleavage yield of avidin increases with irradiation time and becomes saturated at 30 min. Irradiations for longer than 30 min did not increase the product yields.

To locate the cleavage site on avidin, the peptide fragments from the gels were isolated and subjected to amino acid sequencing. The newly generated *N*-terminal sequences of the cleaved fragments were successfully sequenced. *N*-terminal sequencing of both fragments (~9 kDa and ~5 kDa) indicated the residues ARKCS, which is the known *N*-terminal sequence of avidin [24]. However, a minor product with the *N*-terminal sequence of VFTGQ, a sequence internal to avidin, was also observed with the ~5 kDa fragment. From the known sequence of avidin [25], we deduce that the cleavage occurs between Thr 77 and Val 78 (Scheme 2). The crystal structure of avidin shows that this region is proximate to the biotin binding site in the 3D structure of avidin (see Supplementary Information) [26]. The structure of the binding site residues is highly complementary to that of biotin molecule, accounting for specific recognition. If the biotinyl part of Py–biotin binds at the biotin binding site with the pyrenyl chromophore

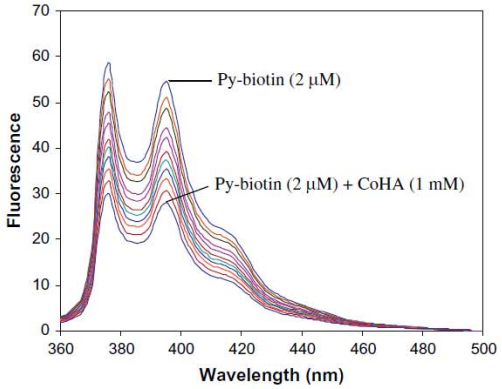

Fig. 2. Fluorescence spectra of Py–biotin (2 μM) with increasing concentrations of CoHA (0–1.0 mM). Excitation wavelength is 342 nm.

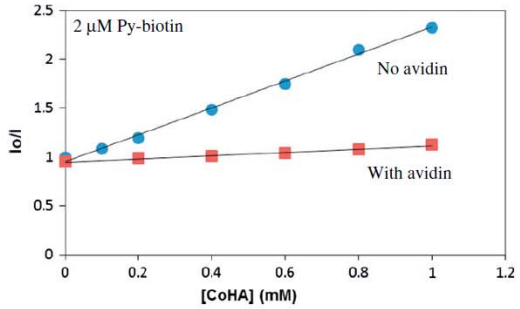

Fig. 3. Quenching plots of the Py–biotin (2 μM) by CoHA, in buffer (closed circles), and in the presence of avidin (10 μM, squares).

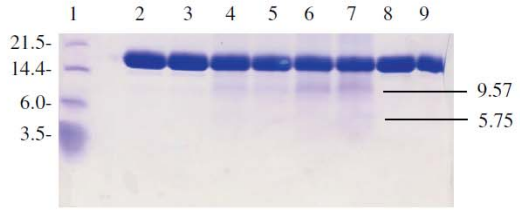

Fig. 4. Site specific photocleavage of avidin by Py–biotin. Lane 1 contained molecular weight markers as indicated (kDa). Lane 2 contained avidin (15 μM). Lanes 3–7 contained avidin (15 μM), Py–biotin (15 μM) and CoHA (1 mM). (Lane 3 was the dark control while samples in lanes 4–7 were exposed to 342 nm radiation for 5, 10, 15 and 30 min, respectively.). Lane 8 contained avidin (15 μM) and Py–biotin (15 μM). Lane 9 contained avidin (15 μM) and CoHA (1 mM). Samples in lanes 8–9 were exposed to 342 nm radiation for 30 min.

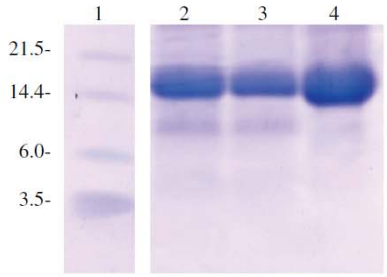

Fig. 5. Inhibition of photocleavage of avidin by d-biotin. Lane 1 contained molecular weight markers as indicated (kDa). Lanes 2–3 contained avidin (15 μM), Py–biotin (15 μM) and CoHA (1 mM), exposed to 342 nm radiation for 15 and 10 min, respectively. Lane 4 contained avidin/biotin mixture (15 μM/60 μM), Py–biotin (15 μM) and CoHA (1 mM), exposed to 342 nm radiation for 15 min.

H_2N-AlaArgLysCysSer…...ArgThr(77) ↓ Val(78)PheThrGlyGln…….COOH

N-terminal fragment — C-terminal fragment (5 kDa)

Scheme 2. Major cleavage site of avidin between Thr 77–Val 78 observed with the *N*-terminal sequence of VFTGQ (5 kDa fragment).

buried at the hydrophobic cavity in the protein near the binding site, then the cleavage could result in the observed region.

From the above results, the pyrenyl chromophore or its singlet excited state (S^1) do not cleave the protein, as indicated by no photocleaved fragments in lane 8 (Fig. 4). The photoproducts can be obtained only when Py–biotin/avidin complex was irradiated in the presence of an electron acceptor, such as CoHA. The mechanism for cleavage of avidin by Py–biotin is expected to be similar to that reported for other pyrenyl probes [22]. The pyrenyl cation radical derived from electron transfer from pyrene singlet excited state to CoHA may be responsible for protein cleavage, as verified in flash photolysis studies, leading to the loss of photocleavage yields.

3.3. Inhibition study of photocleavage of avidin

In the inhibition study, a solution of d-biotin (60 μM) was mixed with avidin (15 μM) for 15 min prior to addition of Py–biotin (15 μM) and CoHA (1 mM) to the avidin/biotin mixture. Interestingly, irradiation of the above mixture solution at 342 nm resulted to the decrease of photocleavage yields (Fig. 5). The inhibition study is consistent with the cleavage site obtained from the amino acid sequencing. d-Biotin may occupy the biotin binding sites on avidin. Therefore, the specific binding site is not available for the incoming Py–biotin.

4. Conclusion

Absorption and fluorescence spectral data clearly indicate the binding of the pyrenyl probe to avidin. The absorption spectra are red shifted when Py–biotin binds to avidin. Fluorescence quenching studies suggest that Py–biotin binding site in avidin is buried in the protein matrix, away from the aqueous phase. Pyrenyl chromophore linked to biotin can be delivered to the specific site on avidin. Upon photoexcitation of pyrene, in the presence of an electron acceptor, the cleavage of avidin can occur in the proximity to the biotin binding site. The formation of pyrenyl cation radical resulted from quenching of pyrene excited state by CoHA is expected to play the important role in the photoreaction as reported previously [13]. The cleavage efficiency of the probe correlates with the accessibility of the protein-bound probe to CoHA. Thus, the accessibility of the pyrenyl chromophore controls efficiency. To improve the accessibility of the protein-bound probe to the quencher, conjugation of a quencher to the probe molecule could be a possible way to enhance the cleavage efficiency in the future studies.

In conclusions, the data in the current studies clearly show that the protein cleavage site can be controlled by appending a desired ligand to the pyrenyl chromophore, thus paving the way for a rational approach to target particular sites on proteins. Therefore, this will be helpful in designing site-specific reagents for the photocleavage of proteins. In addition to biochemical applications, such reagents may be of interest for therapeutic purposes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jphotobiol.2011.04.003.

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