

Article

Search for pterin-binding protein from *Euglena*

Junko Takeda^(1,*), Minoru Nakashima⁽¹⁾, Hiroshi Ueno,⁽¹⁾ Tomoko Mori⁽²⁾.
Mineo Iseki⁽³⁾ and Masakatsu Watanabe⁽⁴⁾

(1) Lab. of Appl. Microbiol. & Biochem., Nara Women's Univ., Nara 630-8506, Japan

(2) National Institute for Basic Biology NIBB Core Research Facilities Functional Genomics
Facility, Okazaki 444-8585, Japan

(3) Dept. Pharmacol. , Toho Univ., Funabashi 274-8510, Japan

(4) Grad. Sch. for Creation of New Photonics Indust., Hamamatsu 431-1202, Japan

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BIAcore analysis with a biopterin (BP)-bound sensor chip could be a powerful tool for the search of pterin-binding proteins. Although preparation of the sensor chip is crucial, a conventional amine coupling procedure is not applicable for the ligand such as BP that has no primary amino group. In this study, we describe the method to introduce the primary amino group onto BP molecule efficiently.

Key words: Biopterin binding protein, biopterin sensor chip for BIAcore, organic synthesis of primary amino group to biopterin.

Introduction

Euglena gracilis, a unicellular photosynthetic flagellate, has served as a model system for studying light-perception and signal transduction in photo-responses

for over a century because it shows various photomovement-responses such as phototaxis, photokinesis and photophobic responses (1, 2). However, the responsible photo-sensor molecules except photoactivated adenylyl cyclase (PAC)(3) for the step-up photophobic response have remained unidentified until recently.

*To whom correspondence should be addressed, telephone:+81-075-702-3417, E-mail:jtakeda-chappy3@ares.eonet.ne.jp

In 2002, Iseki et al. isolated PAC from paraflagellar body (PFB) of *Euglena*. PAC is a heterotetramer composed of two flavin adenine dinucleotide (FAD) binding subunits of alpha and beta chains in two sensors of blue-light-using-FAD (BLUF) domains. The RNA interference (RNAi) analysis indicates that PAC functions as a receptor for the step-up photophobic response and not for the step-down photophobic response. The results suggest a presence of a different photoreceptor for the step-down photophobic response. The action spectrum for the step-up photophobic responses corresponded to the flavin absorption spectrum with two characteristic peaks at 350 nm and 450 nm in the equal height (4), while that for the step-down photophobic response showed two peaks with unequal heights; the peak at 350 nm is higher than that at 450 nm. This indicates the presence of photoreceptor molecules other than PAC, the molecules characterized by having an absorption maximum around 350 nm that was added to the flavin spectrum. One of such substances may be an oxidized biopterin (BP) since the existence of BP-binding protein in *Euglena* was suggested (5,6). However, no method is available in detecting the BP-binding activity with simple and rapid way, which has prevented the identification of BP-binding protein.

We have attempted to isolate the

BP-binding protein from paraflagellar body (PFB)-rich fraction of *Euglena*. For simple and rapid detection of BP-binding activity, we considered the application of BIAcore system that utilizes the surface plasmon resonance. Generally, ligands having primary amino group are immobilized onto BIAcore sensor chips via amine coupling method, whereas those ligands without primary amino group are immobilized via their hydroxyl groups. With many attempts, the method described in BIA direction (7) has not been successful in producing a BP sensor chip. In this study, we introduce a method where the primary amino group attaches to one of the hydroxyl groups of the propyl-side chain of BP in order to apply the conventional amine coupling method.

Materials and Methods

The reagents used were biopterin (BP), 2,2'-ethylenedioxy bis-(ethylamine (diamine) (Sigma-Aldrich, Tokyo, Japan), disuccinimidyl carbonate (DSC), dimethylaminopyridine (DMAP), and dried dimethylsulfoxide (DMSO) (Wako Pure Chemical Industries Ltd, Osaka, Japan). These reagents as well as their stock solutions were stored in a desiccator before use.

Hydrophilic interaction liquid chromatography

BP and the reaction products in DMSO

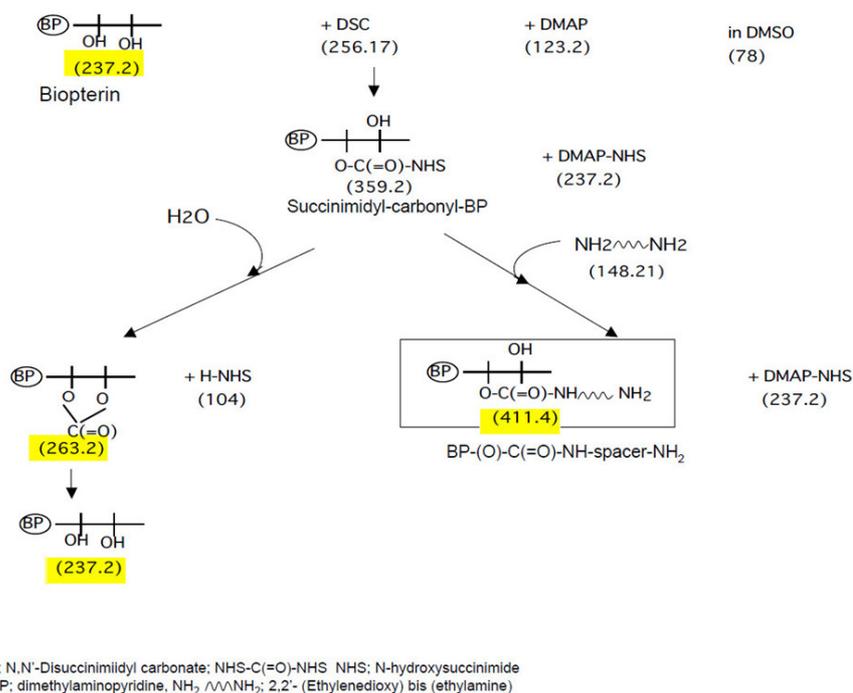


Figure 1 The reaction scheme for the introduction of primary amino group to BP. The product, BP-O-C(=O)-NH-spacer-NH₂ (MW 414.4), is indicated by the solid line box. The compounds confirmed by MS spectrum are marked with yellow.

were fractionated by the hydrophilic (PCHLIC) (Shiseido Co. Ltd Tokyo, Japan) interaction liquid chromatography under the following conditions: The column

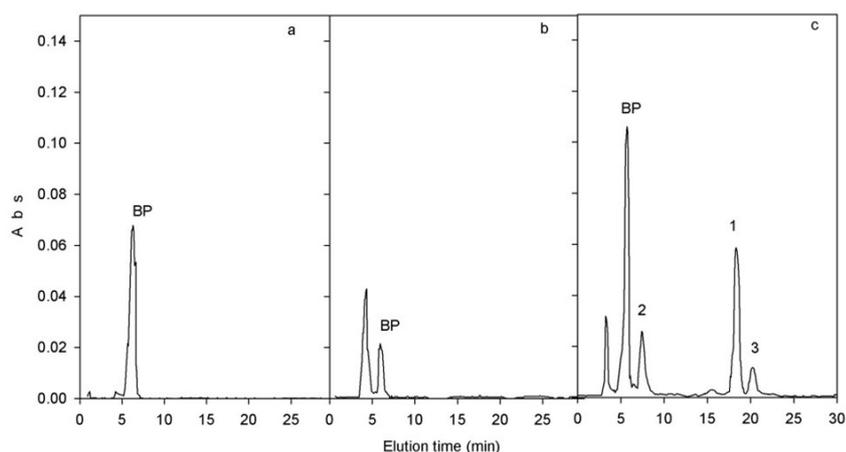


Figure 2 HPLC Elution Pattern of 70% CH₃CN for reaction mixture before and after the reaction. Before: BP (a), DSC and DMAP in DMSO were added to (a) at 2.5 days or more (after the first step reaction completed)(b), and diamine was added to (b) at 30 min (second step reaction completed) (c), respectively. The vertical and horizontal axes show the absorbance at 360 nm and time. Scales show 5 min. intervals

size and eluents are 4.6 mm id x 250 mm, and 10 mmol L⁻¹ HCOONH₄ in 70, 80, or 90% CH₃CN, respectively. The flow rate, temperature, and absorption wavelength are set at 1.0 mL min⁻¹, 28 °C, and 360 nm, respectively.

MS and MS/MS analysis

MS and MS/MS analyses were performed using a Q-TOF mass spectrometer (Waters Co. Milford, USA). The sample was diluted in two-fold with 0.1% HCOOH and applied to the MS spectrometer with a syringe pump.

Results and Discussion

Introduction of the primary amino group to one of the hydroxyl group of BP

The coupling reaction of a primary amino group with an activated hydroxyl is a urethane forming reaction (8). The reaction scheme is illustrated in Figure 1. The products confirmed to have the oxidized BP by the mass spectrometry (MS) are marked with yellow, and the amino group attached BP, (NH₂-spacer-(O)-BP), is indicated by a solid line box. The primary amino group attached BP has MW 411.4 and the molar ratio of BP to diamine is 1:1. The reaction

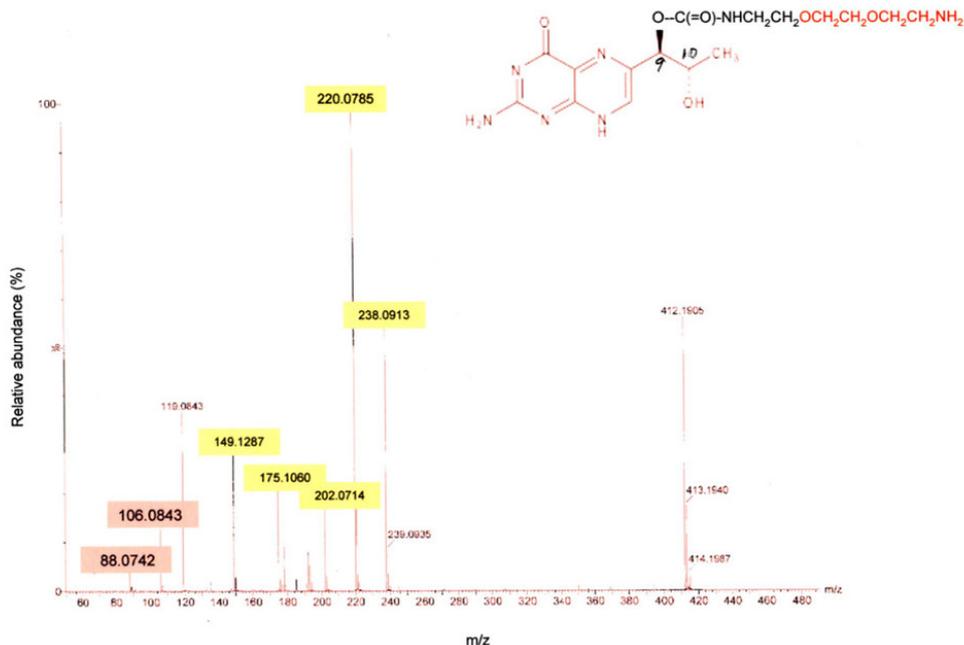


Figure 3 MS/MS spectrum of m/z 412 which picked up from MS spectrum of peak 1.

Fragment peaks of MS/MS for BP are indicated in yellow. The fragments in red are derived from the partial structure $\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{NH}_2$ in red font of inserted figure. Vertical and horizontal axis show the relative abundance and m/z, respectively.

is supposed to proceed in two steps. In the first step, one of the hydroxyl groups of BP was activated with both addition of DSC and DMAP to form a succinimidyl-carbonyl-BP, an activated BP complex. In the second step, the activated BP complex couples to one of the primary amino groups of diamine to form $\text{NH}_2\text{-spacer-(O)-BP}$.

The activation of hydroxyl group requires DSC and the reaction must be carried out in organic solvent since DSC is easily hydrolyzed by water. We chose dry DMSO as a solvent since BP was most soluble in DMSO among the organic solvents examined. In this reaction, 1) one of the two hydroxyl groups on BP must be activated, and 2) the activated BP complex can easily couple to one of the amino groups of diamine. The BP, DSC and DMAP were mixed with their molar ratio of 1:4:1, respectively, and then an excess amount of diamine, approximately 25 times as much as the amount of the BP, was added. After the reaction was completed, no byproducts was detected by the MS analysis for the reaction mixture having the molar ratios of BP to diamine as 1:2 and 2:1.

We have attempted to separate and identify the reaction products by HPLC using a PC HILIC column (Shiseido, Japan). The eluate was monitored at 360 nm as the compounds having the oxidized pterin structure that exhibits the absorption

maximum around 350-360 nm at neutral or alkaline pH. The polarity of the reaction products was expected to be higher than that of BP as the primary amino group was incorporated. BP was eluted at 6.3, 10.5 and 12.0 min when CH_3CN with concentration of 70, 80, and 90%, respectively, was used as an elution solvent. Figure 2 shows the HPLC elution patterns with 70% CH_3CN for (a) BP (b) the reaction mixture of BP, DSC and DMAP after the first step reaction reached at the maximum and remained, and (c) the reaction mixture of (b) after the second step reaction. Figure 2a indicates that BP is eluted at 6 min, Figure 2b shows the appearance of several inseparable peaks as a cluster between 2.5 and 4.0 minutes, and in Figure 2c the cluster disappeared and several peaks appeared. The elution profiles suggest that the cluster is probably the intermediate product having MW 359.2, which is succinimidyl-carbonyl-BP, an activated complex of BP, as shown in Figure 1. The first step reaction proceeded slowly, reached at the maximum after 2.5 days, and remained for more than three months in a desiccator without light. During this reaction, the amount of BP declined while that of inseparable cluster increased with time. Before initiating the second step reaction by adding an excess amount of diamine, a small amount of water was added to the reaction

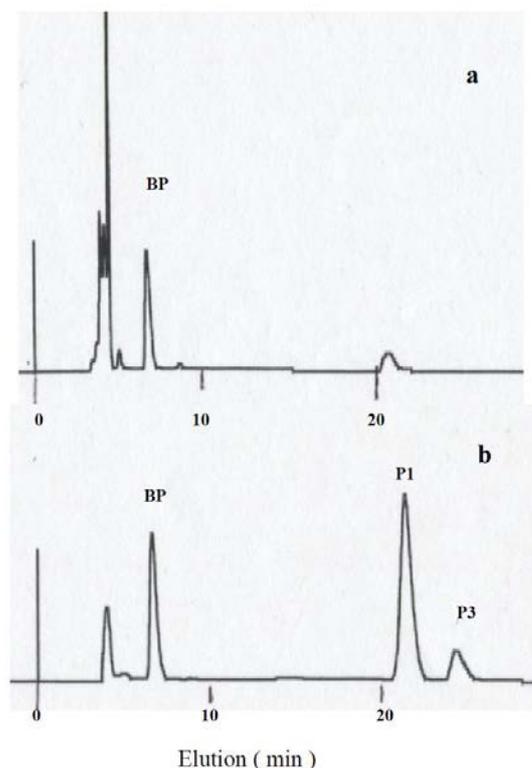


Figure 4 Effects of pH on the second step reaction. The activated BP complex in DMSO was added to diamine solution at pH8 (a) or pH10 (b). After incubation at RT for 30 min, the reaction mixture was analyzed by HPLC.

mixture to prevent further activation of BP. While the activation was inhibited by the presence of a trace amount of water, the reaction product was not hydrolyzed immediately. Only 10% of the reaction product was hydrolyzed when 50% volume of water was added to the reaction mixture and kept overnight in the ice box. The second step reaction proceeded rapidly and was completed within 30 minutes. As shown in Figure 2c, the cluster decreased largely and three distinct peaks appeared.

The peaks are named in descending order in height as peak 1 at 18.5 min, peak 2 at 8 min and peak 3 at 21 min.

The products at the BP peak as well as the peak 1, peak 2 and peak 3 were collected and analyzed by mass spectrometry (MS) to determine their structures. The MS analysis indicated that the mass of the BP peak was 238.0742, which agrees with the theoretical mass of BP, 237.2. A largest peak at 412.1606 agrees with the mass for the expected BP- (O)-spacer-NH₂ (411.4) obtained from peak 1-eluent and other peaks are negligible (less than 10% of the maximum intensity). The peak at 412. was gathered and further analyzed by MS/MS and shown in Figure 3, in which BP and BP fragments are marked with yellow. The peaks marked with red (106.0843, and 88.072) may be originated from the partial structure as indicated with red fonts in the Figure 3 insert. Neither BP nor BP originated fragments were detected from the largest peak at 432.0948 obtained from peak 2-eluent. From peak 3-eluent the largest peak at 412.1838 may be the expected BP- (O)-spacer-NH₂ (411.4). These two products may be produced by the oxygen attach either at C9 or C10.

The MS and MS/MS analysis confirmed BP- (O)-spacer-NH₂ was synthesized as the peak 1 and peak 3 by our described method. We obtained a stable

product, succinimidylcarbonyl-BP (activated BP), in the first step reaction in an organic solvent though the reaction proceeded considerably slowly. Coupling of the activated BP to the primary amino group in the second step reaction was expected to proceed rapidly at high pH. We compared the reaction efficiency at pH 8 and pH 10 (Fig 4). There were no significant changes in the peak of the activated complex whereas the product peaks were barely detected at pH 8 (a). At pH 10, on the other hand, the peak of the activated complex decreased rapidly as the product peaks (peaks 1 and 3) grew, and the byproduct peak (peak 2 in Figure 2) was undetectable (b). The area ratio of the BP and product peaks (peaks 1 and 3) is 1:2.1, indicating a desirably high yield.

Getting the BP derivative having the primary amino group can be purified by a proper preparative column, the BP-sensor chip could be produced by the conventional amine coupling method and used for isolating the BP-binding proteins from *Euglena*.

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References

- (1) Lebert M. (2001) Phototaxis of *Euglena gracilis* - flavins and pterins. In *Comprehensive series in photosciences* (Hader D-P and Lebert M eds.) Vol. 1 Photomovement, pp 297-341, Elsevier.
- (2) Hader D-P, Ntefidou M. Iseki M. and Watanabe M. (2005). In *Light sensing in plants* (Wada M., Shimazaki K. Iino M. eds) pp 223-229, Springer ISBN-4-431-24002-0
- (3) Iseki M., Matsunaga S., Murakami A., Ohno K., Shiga K., Yoshida K., Sugai M., Takahashi T., Hori T. and Watanabe M. (2002) A blue-light-activated adenylyl cyclase mediated photoavoidance in *Euglena gracilis*. *Nature* **415** 1047-1051
- (4) Matsunaga S., Hori T., Takahashi T., Kubota M., Watanabe M., Okamoto K., Masuda K. and Sugai M. (1998) Discovery of signaling effect of UV-B/C light in the extended UV-A/blue-type action spectra for step-down and step-up photophobic response in the unicellular flagellate alga *Euglena gracilis*. *Protoplasma* **201** 45-52
- (5) Brodhun B. and Hader D-P. (1990) Photoreceptor proteins and pigments in the paraflagellar body of the flagellate

- Euglena gracilis*. *Photochem. Photobiol.* **52** 865-871
- (6) Brodhun B. and Hader D-P. (1995) A novel procedure to isolate the chromoproteins in the paraflagellar body of the flagellate *Euglena gracilis*. *J. Photochem. Photobiol.* **B 28** 39-45
- (7) Biacore^R Sensor Surface Handbook. Edition October 2003 (Version AA)
- (8) Yang M., McGovern M. E. and Thompson M. (1997) Genosensor technology and detection of interfacial nucleic acid chemistry. *Anal. Chimica Acta* **346** 259-275

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