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G. 知的財産の出願・登録状況

1. 特許取得

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2. 実用新案登録

なし

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Chemiluminescence detection of amino acids using an Edman-type reagent, 4-(1′-cyanoisoindolyl)phenylisothiocyanate

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Abstract

The 4-(1′-cyanoisoindolyl)phenylisothiocyanate (CIPIC) could be used as a chemiluminescence (CL) Edman-type reagent. Firstly, CL reaction conditions such as pH, buffer types, H_2O_2 concentration and organic solvents were optimized in detail. Secondly, the CL sensitivity of the derivative of Ala was compared with fluorescence and absorbance detections. Thirdly, the structures of the CL intermediate and emitter were elucidated by liquid chromatography–mass spectrometry (LC–MS) and NMR of CIPIC and its analogues after their CL reactions. Consequently, the thiohydantoin derivatives for 16 kinds of amino acids were separated and sensitively detected by CL with lower detection limits of 0.3–0.8 pmol at a signal-to-noise ratio of 3, after the coupling reaction of amino acids with CIPIC and their cyclisation reaction.

Keywords: Chemiluminescence detection; Amino acid; Derivatisation; Edman-type reagent; CIPIC

1. Introduction

With the complete sequence of human genome, the whole genomic and proteomic expressions have been recently investigated. It will provide various informations about the coordinate regulation among many genes since almost all cell activation or phenotypes are the sum of a series of molecular and biochemical events interacting with each other in a complex fashions. It is well known that gene expression is controlled by numerous fundamental and selective protein–protein, protein–DNA, protein–RNA and protein–ligand interaction. Therefore, the post-genomic era has been a rapidly increasing need and desire to enhance and support genomic studies with proteomic data. The medical rationale behind analyzing proteins is to improve our understanding of normal and disease processes. Consequently the development of protein technology is beginning to move forward rapidly. Most importantly, the ability to characterize the composition and amino acid sequence of proteins is highly desirable.

However, phenylisothiocyanate (PITC), the Edman reagent, is still the most widely used for the determination of the amino acid composition and sequence of protein and peptides [1–5]. The Edman procedure consists of three steps: a coupling reaction with Edman reagent; a cyclisation/cleavage reaction in the presence of anhydrous acid and generation of amino acids thiazolinone derivatives; and a conversion reaction to stable thiohydantoin derivatives. The reactivity with PITC is high and the repetitive yield is good (more than 95%). However, the sensitivity of UV absorbance detection for the amino acid derivatives with PITC is fairly low compared with generally used laser-based fluorescence method for the detection of DNA sequence. Therefore the main drawback of this UV detection method is that at least 100 pmol peptide

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or protein is generally needed for the amino acid sequencing due to its comparative low sensitivity. It is often unacceptable, especially for some precious protein or peptide samples.

In order to solve this problem, several other Edman-type reagents have been synthesized, such as UV-based 2-*p*-isothiocyanophenyl-3-phenylindone [6], 4-*N*,*N*-dimethylaminoazobene-4-isothiocyanate [7,8], 4-(*N*-*tert*-butyloxycarbonylaminomethyl) phenylisothiocyanate [9], fluorescence-based fluoresceinisothiocyanate [10], 4-(*N*,*N*-dimethylamino)-1-naphthylisothiocyanate [11], 4-(*N*-1-dimethylamino-napthalene-5-sulfonylamine)phenylisothiocyanate [12], 4-(2-phenanthra-[9,10-*d*] oxazolyl)phenylisothiocyanate [13], 4-(3-isothiocyanatopyrrolidine-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [14], and chemiluminescence (CL)-based 4-isothiocyanatophthalhydrazide [15]. However, still two main drawbacks existed in these methods: the repetitive yield in the Edman procedure is low and the degradation products sometimes interfere with the detection of thiohydantoin-amino acids. Recently, Imai and co-workers developed another two benzofurazan Edman reagents: 7-[(*N*,*N*-dimethylamino)sulfonyl]-2,1,3-benzoxadiazol-4-isothiocyanate [16–18], and 7-methylthio-4-(2,1,3-benzoxadiazolyl)isothiocyanate [19]. Although thiohydantoin-amino acids fluoresced weakly and thiazolinone-amino acids fluoresced strongly, free amino acids were observed due to the decomposition of unstable thiazolinone. Compared with previous methods, however the sensitivity is much higher with a detection limit of 50 fmol–1 pmol.

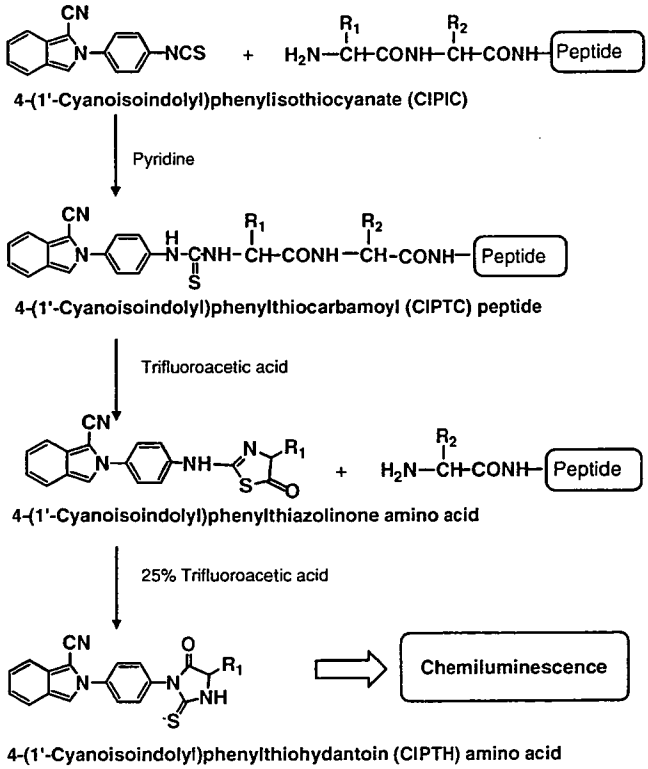

Fig. 1. Edman degradation with CIPIC for peptide sequence.

To overcome the above disadvantages, we reported a novel fluorescent Edman reagent, 4-(1′-cyanoisoindolyl) phenylisothiocyanate (CIPIC) [20]. CIPIC was found to be of high reactivity with amino acid in the Edman procedure. It is well known that CL has been exploited with a wide range of applications in different fields such as biotechnology, pharmacology, molecular biology, and clinical and environmental chemistries. Specifically CL-based analysis offers a simple, low-cost, sensitive means of measuring a variety of compounds. Thus CIPIC was further investigated to see whether it is possible to tune as a sensitive CL-type Edman reagent. Our results show that CIPIC could induce a strong CL after mixing with H_2O_2 in an alkaline solution, and thus can be used for the CL detection of amino acids (Fig. 1).

2. Experimental

2.1. Apparatus

Batch CL measurements were made by using a luminescence reader (BLR-201, Aloka, Japan). Fluorescence and UV measurements were done by employing Hitachi 650-10 and Shimadzu UV-240, respectively. LC system consisted of JASCO PU-980 pump and UV-970 detector. LC–MS measurements were carried out by using M-1000 quadruple mass spectrometer (Hitachi, Japan) with atmospheric chemical ionization at 200 °C, nebulizer temperature at 370 °C, filament current at +8 μA, drift voltage at 110 V and focus voltage at 60 V.

2.2. Materials and chemicals

Buffer solutions for pH 9–13 for CL reaction were prepared by mixing 0.1 M boric acid and 0.1 M sodium borate, 0.1 M boric acid and 0.1 M triethylamine, or 0.1 M $NaHCO_3$ and 0.1 M NaOH. Buffer solution (pH 5.0) for liquid chromatography (LC) was made by mixing 0.1 M acetic acid and 0.1 M triethylamine. CIPIC, CIPIC-thiocarbamoyl-Ala (CIPTC-Ala) and CIPIC-thiohydantoin (CIPTH-Ala) stock solutions, and their working solutions were prepared in acetonitrile. All other reagents and solvents were of analytical or guaranteed reagent grade.

2.3. Synthesis of CIPIC, CIPTC-Ala and CIPTH-Ala

CIPIC was synthesized as reported previously [20]. In order to synthesize CIPTC-Ala, 50 ml of 80 mM L-Ala in acetonitrile and water (4:1, v/v), 10 ml of 10 mM CIPIC in acetonitrile and 50 ml of pyridine and acetonitrile (2:3, v/v) were reacted at 80 °C for 20 min. After cooled to room temperature, 100 ml of 25 mM sodium phosphate buffer (pH 8.5) and 300 ml chloroform were added into the reaction mixture. The aqueous phase was separated from organic phase and kept at 4 °C (solution A). Solution A (80 μl each) was subjected to reversed-phase LC and CIPTC-Ala frac-

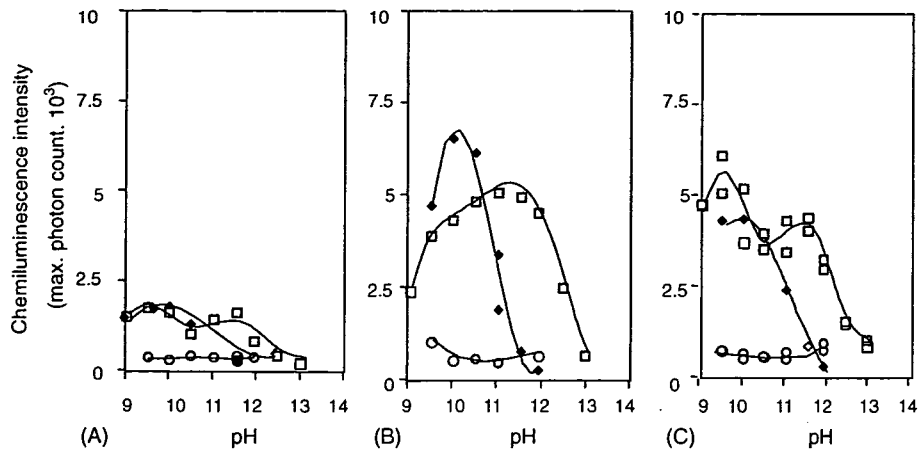

Fig. 2. Effects of pH of several buffers on the chemiluminescence reaction with (A) CIPTH-Ala, (B) CIPTC-Ala, and (C) CIPIC. Buffers: (□) sodium-borate; (◇) triethylamine-borate; (○) $NaHCO_3$-NaOH.

tion (ca. 20 mg) was obtained. To purify CIPTH-Ala, solution A was mixed with 200 ml of 2.5 M HCl, and then heated at 80 °C for 20 min for converting CIPTC-Ala to CIPTH-Ala. The resulting solution was cooled to room temperature and 400 ml chloroform was added. The chloroform phase was concentrated, and the precipitate was recrystallised in acetonitrile and water. CIPTH-Ala (ca. 150 mg) was obtained.

2.4. CL detection procedures

Light producing reactions were carried out in 12 mm × 75 mm disposable culture tubes containing 100 μl of CIPIC, CIPTC-Ala and CIPTH-Ala at different concentrations. Portions of 100 μl of 0.1 M borate buffer (pH 9.6), and 50 μl of acetonitrile were added, and the tubes were placed in the luminescence reader. A 50 μl portion of 1.5 M H_2O_2 was injected and the cover was closed to initiate CL. The signal was displayed and integrated for 30 s. The progress of the CL was monitored on a recorder connected to the luminescence reader.

2.5. Fluorescence and absorbance detections

Fluorescence intensity was detected at Ex = 350 nm and Em = 425 nm for CIPIC in acetonitrile, and at Ex = 350 nm and Em = 410 nm for CIPTH-Ala in acetonitrile. UV absorbance was measured at 275 nm for CIPIC and 255 nm for CIPTH-Ala.

2.6. Synthesis of N-(4-acetylaminophenyl) phthalimide (product B in Fig. 7)

Twelve millilitres of 72 μM phthalic anhydride in acetonitrile and dimethylformamide (2:1, v/v) were mixed with 6 ml of 36 μM *p*-aminoacetanilide in acetonitrile and dimethylfor-

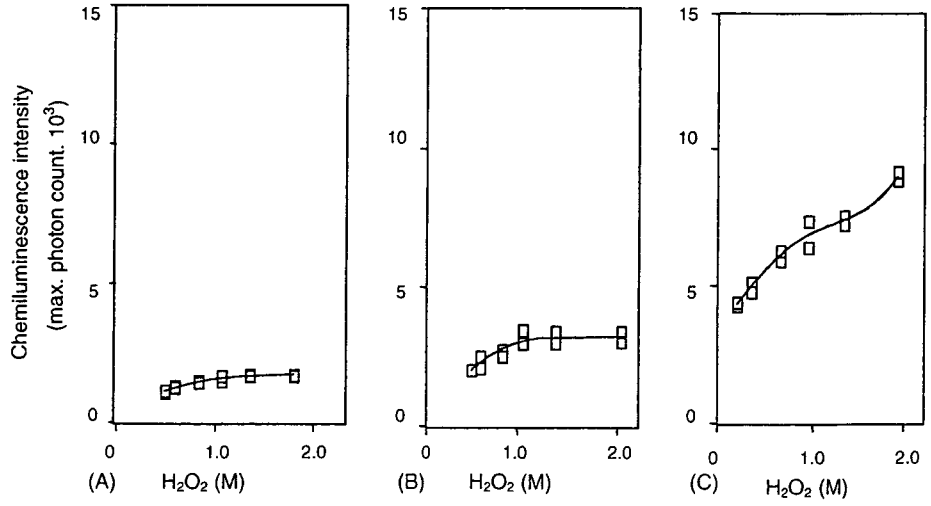

Fig. 3. Effects of concentration of hydrogen peroxide at pH 9.6 and acetonitrile on the chemiluminescence reaction with (A) CIPTH-Ala, (B) CIPTC-Ala, and (C) CIPIC.

mamide (2:1, v/v), and then stirred at room temperature for 30 s. White precipitate (ca. 1.7 g) was formed and filtered. The precipitate (450 mg) was dissolved in 120 ml of tetrahydrofuran and dimethylformamide (1:1, v/v), 6 ml of conc. HCl was added and then the reaction was carried out at 80 °C for 40 min. Water (240 ml) was added into the reaction mixture and then ice-cooled overnight. The product was filtered and recrystalised in tetrahydrofuran (product B left in Fig. 7).

2.7. LC conditions

A reversed-phase column of Cosmosil 3C (100 mm × 4.6 mm i.d., particle size 3 μm) (Nacalai tesque, Japan) was used. The separation of the CIPIC conjugated amino acids was carried out with a mobile phase of acetonitrile (45–90%) and 0.1 M acetic acid–0.1 M triethylamine mixture (pH 5.0), and the flow-rate was set at 0.5 ml/min. The column eluate was first monitored by fluorescence and then introduced into the postcolumn reactor. In the system, 1.0 M H_2O_2 and 0.1 M sodium borate buffer (pH 11) were added to the eluate stream with two reagent-delivery pumps at each flow-rate of 0.23 ml/min. CL was monitored by intelligent CL detector (JASCO 825-CL, Japan).

3. Results and discussion

3.1. Optimum CL conditions

It is well known that the separation technique plays a key role for the detection of different analytes in complex matrixes. Reversed-phase LC has become dominant as a potential tool for the separation and quantification of amino acids or peptides. Acetonitrile, methanol and tetrahydrofuran (THF) are generally used as organic solvents for elution. Therefore it is very important to see whether these commonly used organic solvents were suitable to be used in the current CL reactions. Surprisingly, we found that these three organic solvents, i.e. acetonitrile, methanol and THF, all enhanced the CL intensity in the current CL reactions. These results indicated that CIPIC-based CL Edman reagent is wholly compatible with currently used reversed-phase LC system. For the detection of CIPIC-conjugated amino acids, H_2O_2 solution was simply mixed with the separated eluates and CL was monitored on a recorder. Acetonitrile was used in the following experiments.

It was found that CL intensity was greatly affected by the reaction pH or buffer type. Indeed CL intensity was much higher in either borate-triethylamine or sodium borate buffer than that in $NaHCO_3$–NaOH buffer (Fig. 2). Thus sodium borate buffer was chosen for CL reactions. As shown in Fig. 3, the CL intensity increased when the concentration of H_2O_2 was increased.

Also as shown in Fig. 4, the shape of the kinetic profile of CL emittance is greatly affected by the reaction pH. At pH < 9.6, the signal is small but the signal decays slowly. At pH > 9.6, the signal is much larger, but it decreases quickly after reaching the maximum. The maximum signal was the highest at pH 9.6 in the borate buffer solutions. Therefore, pH 9.6 of 0.1 M sodium borate buffer and 1.5 M H_2O_2 were selected in the following experiments.

3.2. Comparison of calibration curves and detection limits by UV, FL and CL detections

Since CIPIC and related conjugates all have UV absorbance, fluorescence (FL) and CL emission, thus it is worth to compare with detection installation to provide a best choice. The concentrations of 25–250 nM CIPTH-Ala derivatives were thus exemplified to compare these three detection techniques. As shown in Fig. 5, by using UV detection, a calibration curve is obtained with a poor accuracy due to large error caused by electrical noise. Comparatively, a calibration curve was obtained with a good accuracy by either using FL

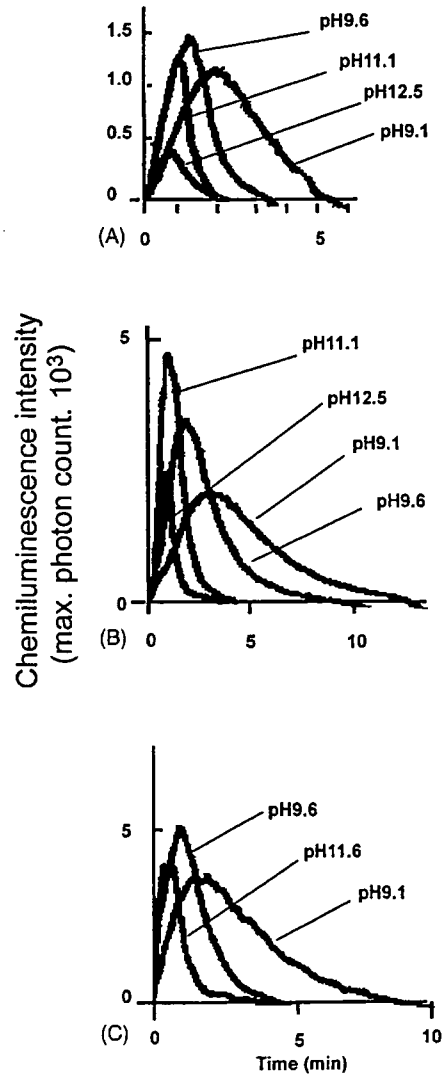

Fig. 4. Time-course of chemiluminescence development from (A) CIPTH-Ala, (B) CIPTC-Ala, and (C) CIPIC depending on pH.

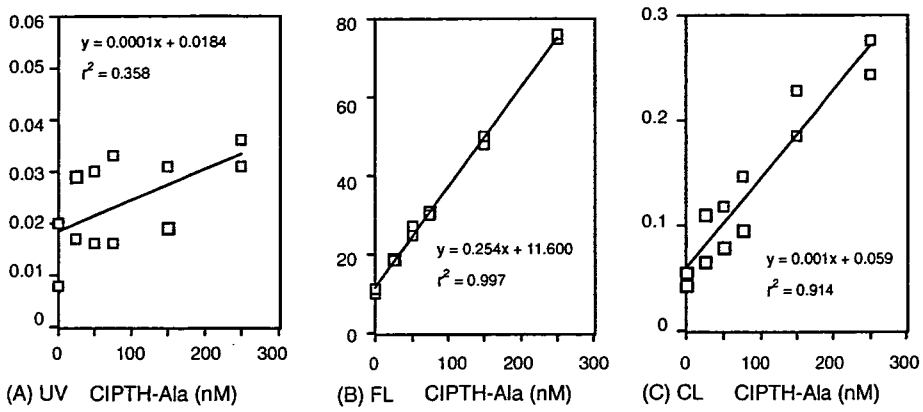

Fig. 5. Calibration graphs of 25–250 nM CIPTH-Ala obtained by (A) UV absorption, (B) fluorometric, and (C) chemiluminometric methods.

or CL detection. These results indicated that CL or FL detection was 20 times more sensitive than UV absorbance.

3.3. Structure elucidation of CL intermediate and emitter

The CIPIC analogues, 4-(1′-cyanoisoindolyl)acetanilide (CIAA) and 4-(1′-cyanoisoindolyl)aniline (CIA) were exemplified to elucidate possible structure of CL intermediates and emitters. CIAA first reacted at room temperature with H_2O_2 in borate buffer (pH 8.0), and then the reaction mixture at a reaction time was separated by reversed-phase LC equipped with UV detector. As shown in Fig. 6, CL reactant and products are eluted within 50 min and well separated from each other. Importantly, peak A was first increased with the reaction time, and then decreased with the increase of peak B, while CIAA and CIA were decreased with the reaction time. The same phenomenon was observed for both CL reactions. These facts indicated that peak A was possible intermediate of this CL reaction and peak B was the CL emitter.

Subsequently, LC–MS was then used to further study the mass spectrometric analysis of these CL intermediates and emitters. For CIAA reaction, both product A (intermediate) and product B (emitter) had a molecular weight of 280. For CIA reaction, both products A and B had a molecular weight of 238. The mass difference between these products obtained by CIAA- and CIA-CL reactions was 42. It should be mentioned that the difference of CIAA and CIA structures is one substituting group with $-NHCOCH_3$ for CIAA and $-NH_2$ for CIA, and its mass difference is exactly 42. This clearly indicates that the CL emission was occurred by the oxidation in the cyanoisoindole part of CIAA and CIA.

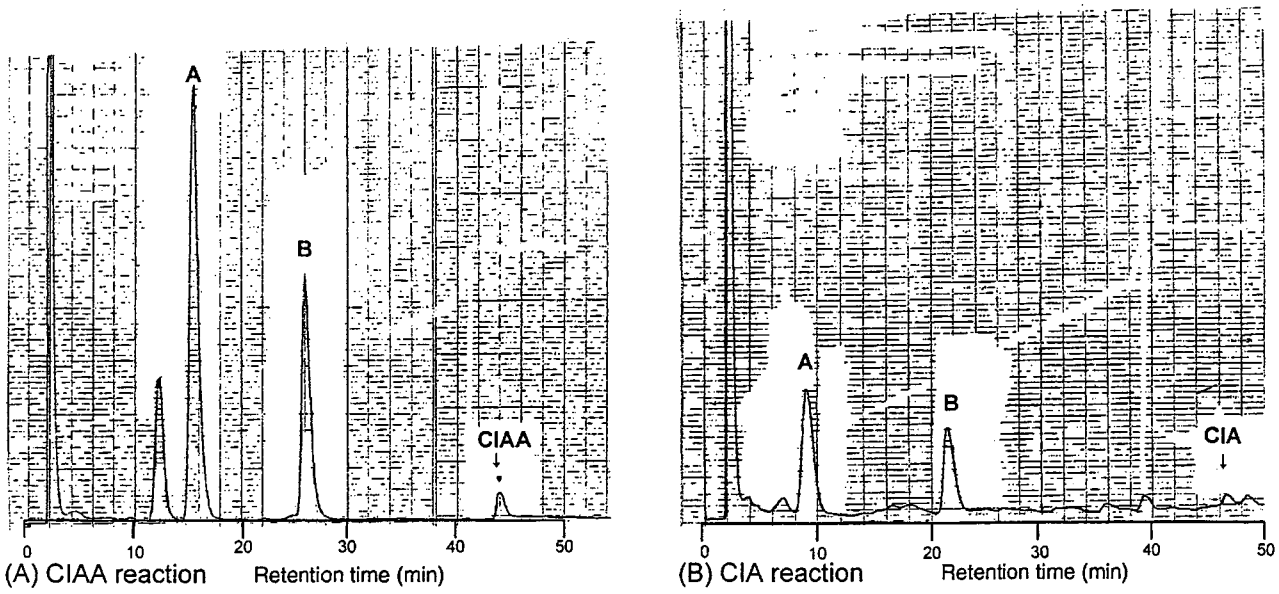

Fig. 6. LC separation and UV detection of chemiluminescent reaction mixtures with (A) CIAA and (B) CIA. LC conditions: column = Cosmosil $3C_{18}$; mobile phase CH_3CN (15–40%) + 0.1 M CH_3COOH–triethylamine, pH 4.9 (10%) + H_2O (75–50%) for 40 min and 50% CH_3CN after 50 min.

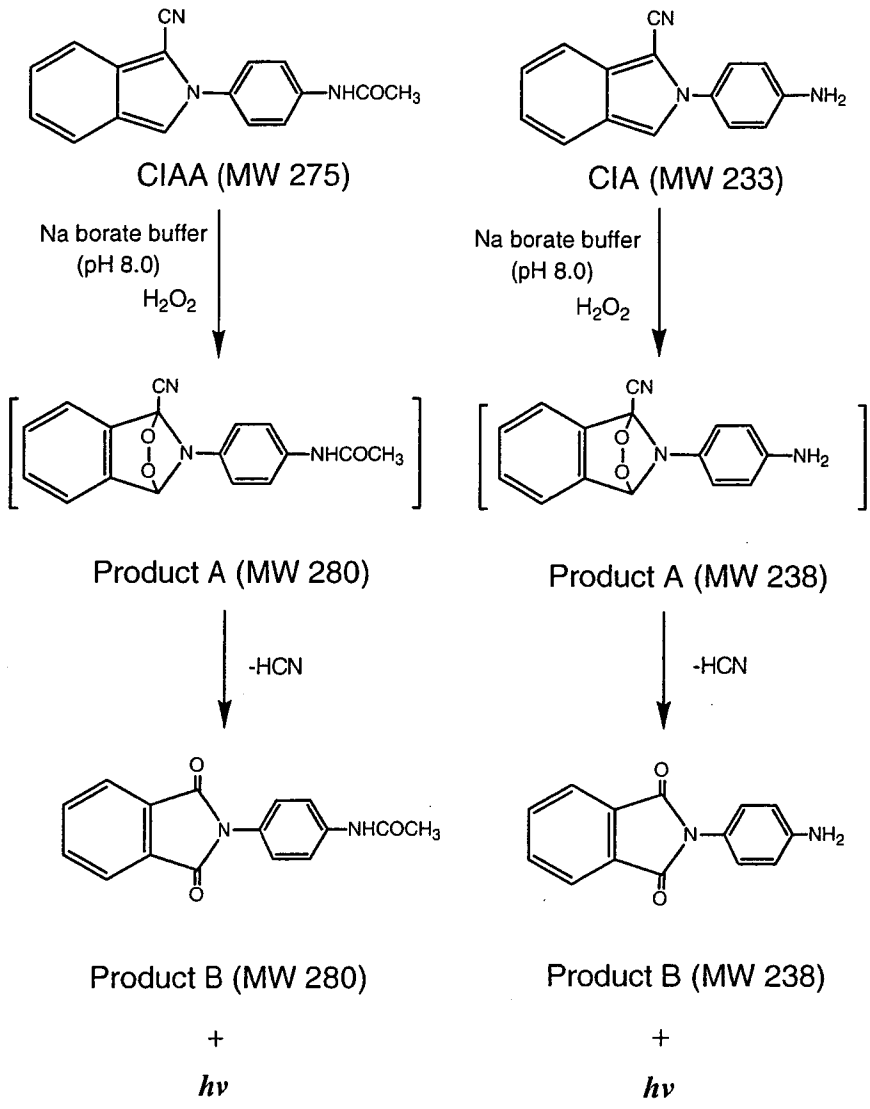

Fig. 7. Products estimated from CIAA and CIA in their mixtures of chemiluminescence reaction.

From the data, possible reaction scheme is shown in Fig. 7. CIAA or CIA first formed a six-membered ring of peroxide (intermediate), and then by the breakage of –O–O– bond, the excited state of phthalimide derivative (emitter) was produced, yielding emission of light.

In order to further confirm that CL emitter was the phthalimide derivative, the product B in Fig. 7 was synthesized from phthalic anhydride and *p*-aminoacetanilide as described in Section 2. The product (melting point, 276 °C) was *N*-(4-acetylaminophenyl)phthalimide, and then its structure was characterized by ^{1}H NMR and ^{13}C NMR. Three or seven peaks from aromatic regions of 7.3–7.9 ppm in the ^{1}H NMR spectrum and 120–140 ppm in the ^{13}C NMR spectrum were observed, respectively. We believed that the data show the symmetric structure of product B. Subsequently CL emitter of CIAA reaction (peak B in Fig. 6) was collected and dried, and then characterized by ^{1}H NMR. It was found that the same ^{1}H NMR spectrum was observed for the peak B as the CL emitter of CIAA reaction. Furthermore, the same retention time was observed for the peak B in Fig. 6 and the synthetic compound (the product B) by the same RP–LC conditions. Overall, it was confirmed that CL emitters of CIAA, CIA and CIPIC are phthalimide derivatives.

3.4. Separation of CIPTH-amino acids

For the detection of CIPTH-amino acids, H_2O_2 solution and borate buffer were simply mixed with the column eluates after separation of 21 amino acid derivatives, and CL was then monitored on a recorder. As shown in Fig. 8, all CIPTH-amino acids were eluted within 30 min. Sixteen CIPTH-amino acids were well separated and were monitored by both FL and CL. Peaks 11, 19, and 20 were also confirmed by directly injecting free CIPIC after derivatization or thiocarbamoyl amino acids into the column. Detection limits of these amino acids at 3 of signal-to-noise ratio were

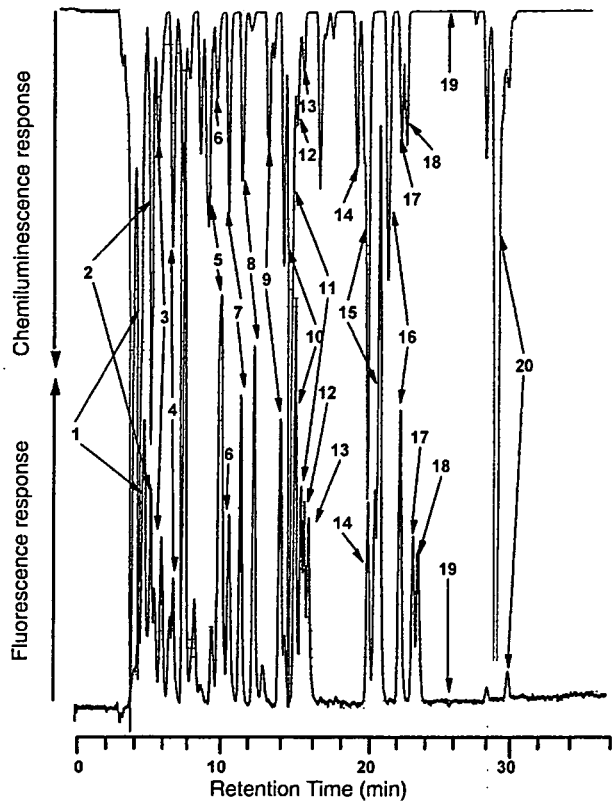

Fig. 8. CL and FL chromatograms of the CIPTH-amino acids. Peaks: (1) Glu and Asp; (2) Arg; (3) Cys; (4) Lys; (5) His and Gln; (6) Asn; (7) Ser; (8) Thr; (9) Gly; (10) Hyp; (11) byproduct from CIPIC; (12) Ala; (13) Tyr; (14) Met; (15) Val, Pro and Trp; (16) Phe; (17) Ile; (18) Leu; (19) CIPIC; (20) CIPIC-byproduct.

0.3–0.8 pmol/injection. The detection limit of CIPTH-amino acids was almost same as those (pico or subpicomole) of hydantoin derivatives formed by other FL reagents [10–14].

4. Conclusion

This work shows that our developed fluorescent Edman-type reagent could be tuned as a sensitive CL Edman reagent. CL reaction conditions were optimized, and the structures of CL intermediate and emitter were elucidated and confirmed by using LC, LC–MS and NMR. Furthermore, this novel CL reaction was wholly compatible with reversed-phase LC for the separation of amino acid conjugates since commonly used organic solvents enhanced CL intensity. However the sensitivity of the present CL detection of CIPTH-amino acids was not much higher than the FL detection. It means that FL detection is simpler and more convenient than this CL detection. Next step, we will synthesize the modified CIPIC reagents as more sensitive CL reagents, and develop the Edman-degradation conditions for the determination of amino acid sequence of protein.

Acknowledgement

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第16章 タンパク質と核酸の標識

16.1 はじめに

タンパク質や核酸の標識は，プロテオミクス研究やゲノム研究において，必要不可欠なものである．タンパク質の標識は，病因や生命現象の解明，イムノアッセイによる微量物質の検出などに応用されており，核酸の標識は，DNA プローブとして，DNA の塩基配列決定，遺伝病の診断，ウイルスや病原菌の検出，親子鑑定など，多くの分析手法に活用されている．これらタンパク質や核酸を標識する物質としては，さまざまなものが使用されているが，検出法の原理において，直接標識物質と間接標識物質に大別することができる．

直接標識物質は，それ自体が，検出シグナルを与える活性をもっており，直接，標識した分子を検出できるもので，蛍光物質や放射性同位元素，酵素などがある．これに対して，ビオチンやタグタンパク質(16.2.3 項参照)などは，検出シグナルを与えないので直接検出することはできないが，これらに，直接標識されたアビジンや抗体を特異的に結合させることによって，間接的に目的タンパク質や核酸を検出できる．このようなビオチンやタグタンパク質などの物質を間接標識物質という．

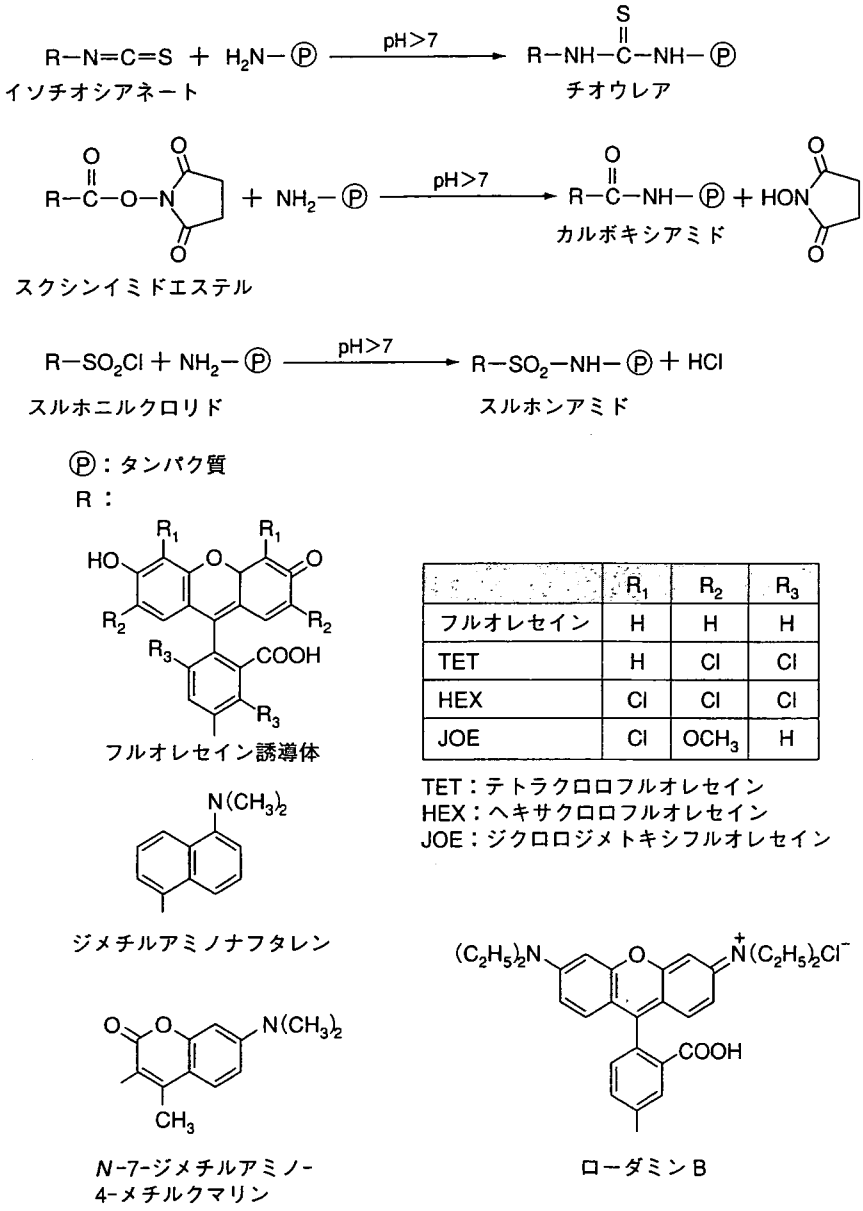
16.2 タンパク質の標識

タンパク質の標識には，標識物質に蛍光物質を用いた蛍光標識，酵素と架橋剤を用いた酵素標識，遺伝子組換え法によるタグタンパク質の標識または安定同位体の標識などがあり，標識されたタンパク質は，イムノアッセイ(immunoassay)，タンパク質の構造解析，機能解析，動力学解析などに用いられる．

イムノアッセイ
抗原(分析対象物)に対する特異的な抗体を用いて，分析対象物と抗体間の抗原抗体反応を利用した測定法．

16.2.1 蛍光物質による標識

タンパク質の蛍光物質による標識は，標識したタンパク質の生体内や細胞内の局在を蛍光顕微鏡下で直接観察できるなどの利点があり，細胞レベルの研究にお



	R_1	R_2	R_3
フルオレセイン	H	H	H
TET	H	Cl	Cl
HEX	Cl	Cl	Cl
JOE	Cl	OCH_3	H

TET：テトラクロロフルオレセイン
HEX：ヘキサクロロフルオレセイン
JOE：ジクロロジメトキシフルオレセイン

図 16.1　アミノ基の蛍光標識反応と蛍光団

いて広く利用される．代表的な標識物質としては，タンパク質のアミノ基と反応するイソチオシアネート，スクシンイミドエステル，スルホニルクロリド(図16.1)など，またはチオール基と反応するハロゲン化アルキル，マレイミド(図16.2 a)などがあり，それらの蛍光団として，フルオレセイン誘導体，ローダミン誘導体，クマリン誘導体などが使用されている．また，チオール基と結合する水銀原子を導入した蛍光物質(図 16.2 b)もタンパク質の標識物質として用いることができ，酵素の反応機構の研究などに利用されている．

16.2.2　酵素による標識

酵素の中には，その酵素反応により，発色性または発光性の生成物を与えるも

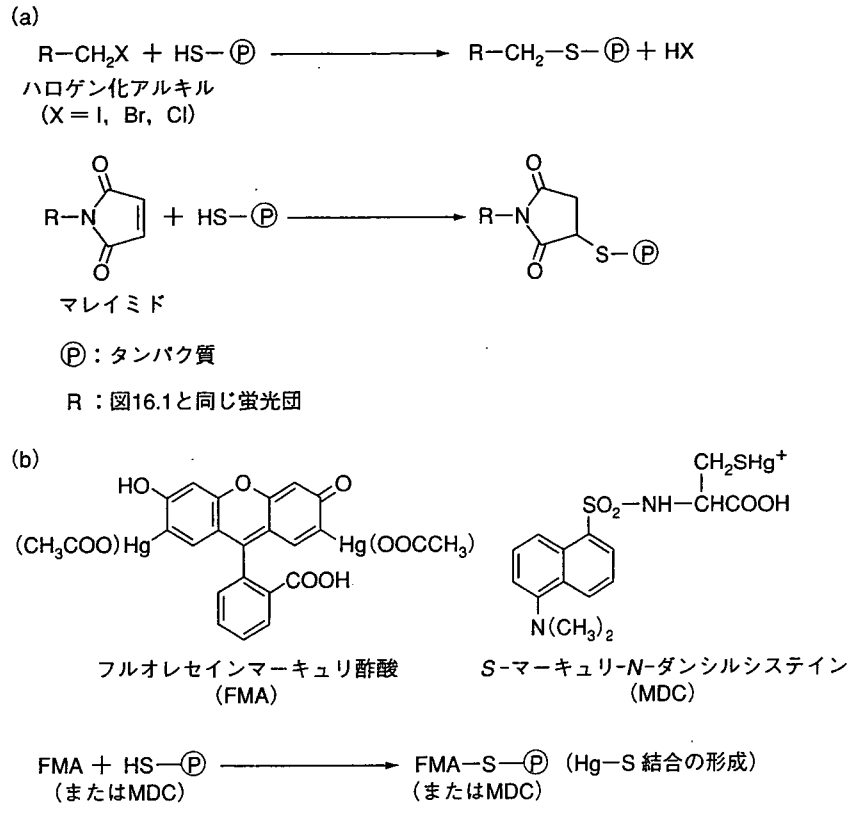


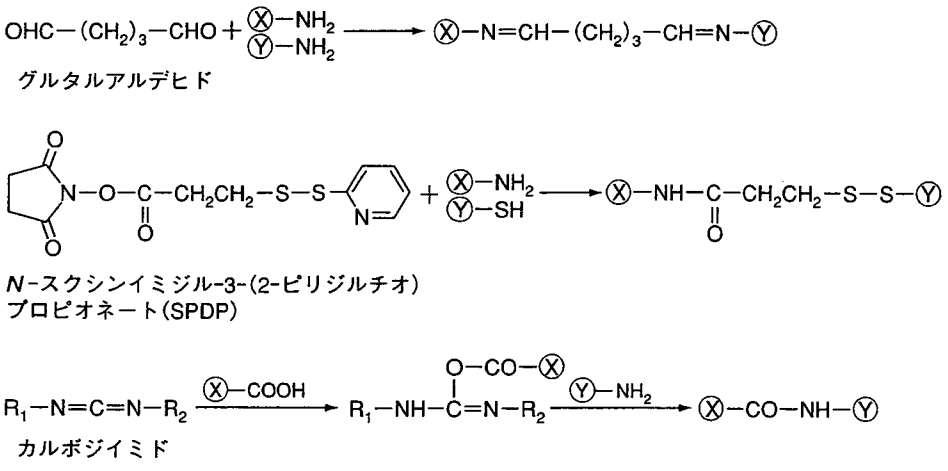
図 16.2　(a) チオール基の蛍光標識反応，(b) 水銀含有蛍光物質

表 16.1　酵素標識に用いられる酵素と発色基質

標識酵素	基質	吸光波長(nm)
ペルオキシダーゼ	5-aminosalitylic acid	450
	o-phenylenediamine(OPD)	492
	tetramethyl benzidine(TMB)	450
アルカリホスファターゼ	*p*-nitrophenylphosphate	410
β-ガラクトシダーゼ	*o*-nitrophenyl-β-D-galactoside	420
グルコースオキシダーゼ	β-D-glucose + POD + TMB(OPD)	450(492)

のがある．この性質を検出法に利用するために，酵素が標識物質として用いられる．このような酵素としては，ペルオキシダーゼ，アルカリホスファターゼ，β-ガラクトシダーゼ，グルコースオキシダーゼなどがあり，被標識物質とアッセイ原理により，標識用の酵素と基質が選択される(表 16.1)．酵素による標識では，蛍光物質の場合と同様に，被標識分子内のアミノ基やチオール基に架橋剤を用いて酵素を結合させる．

架橋剤は，一般に 2 個の官能基(A，B)をもつ試薬 A-R-B で，R を介して二分子間を連結する(図 16.3)．架橋剤には，タンパク質と標識物質のアミノ基同士を結合させるものとしてグルタルアルデヒド，チオール基とアミノ基を結合させるものには活性エステル，アミノ基とカルボキシル基を結合させるものとしてカルボジイミドなどがよく用いられる．架橋剤を用いて酵素などの分子量の大きい



Ⓧ, Ⓨ：一方は被標識タンパク質，残りの一方は酵素などの標識物質

図 16.3　架橋剤によるタンパク質の標識反応

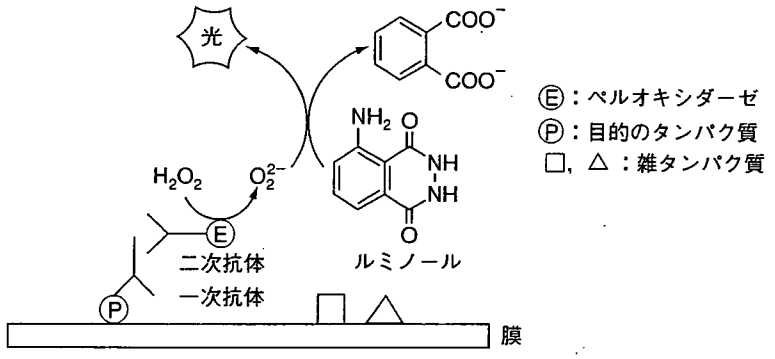


図 16.4　イムノアッセイにおける酵素標識抗体の利用

一次抗体は目的タンパク質を認識する抗体であり，二次抗体は一次抗体を認識する抗体で，酵素(ペルオキシダーゼ)で標識している．この化学発光検出系では，過酸化水素存在下，ペルオキシダーゼの触媒作用によってルミノール自体が酸化されて励起状態になる．そこから基底状態に戻る際にエネルギーを光として放出する．これを化学発光という．これに対して，基底状態にある物質が光のエネルギーを吸収して励起状態になり，その後，励起状態から基底状態に戻る際に，エネルギーを光として放出する場合がある．この光を蛍光またはりん光という．

物質により標識する場合，標識されるタンパク質の本来の機能を損うことがあるので注意を要する．

架橋剤による標識は，抗体を酵素で標識するのに広く用いられており，酵素免疫測定法(enzyme linked immuno sorbent assay；ELISA)や組織染色などのイムノアッセイに広く応用される．図 16.4 は，目的とするタンパク質の検出に酵素標識抗体を利用したものを示したものである．まず，膜上に目的のタンパク質を固定化した後，一次抗体，二次抗体と順に反応させ，ペルオキシダーゼの酵素活性によりルミノールを化学発光させ，目的のタンパク質を検出する．

16.2.3　遺伝子を組換え標識

大腸菌や無細胞発現系により，組換えタンパク質をつくることができる．このとき，^{13}C や ^{15}N を発現系に加えることで，安定同位体で標識したタンパク質を得ることができる．このような安定同位体標識タンパク質は，NMR による構造解析などに利用される．

NMR では，天然に多く存在する ^{12}C や ^{14}N が観測し難いため(第 12 章参照)，核スピンを有する ^{13}C や ^{15}N を用いる．

また，緑色蛍光タンパク質(green fluorescence protein；GFP)のような蛍光タ

ンパク質を対象とするタンパク質に遺伝子レベルで融合することで，蛍光タンパク質と融合したタンパク質を作製することができる．これは，蛍光顕微鏡と組み合わせることで，細胞内における対象タンパク質の動態が直接観察でき，その局在検出や機能解析に利用される．GFPのような目印となるタンパク質をタグタンパク質(tag protein)といい，目的タンパク質の検出や精製に用いられる．

GFPのほかに，タグタンパク質として適応されているタンパク質としては，グルタチオン-*S*-転移酵素，マルトース結合タンパク質，ポリヒスチジン，ヘマグルチニンなどがある．一般的に，融合タンパク質は，本来のタンパク質の機能を保持していることが多いが，まれに機能が消失または変化している場合があり，注意を要する．

例題 16.1　タンパク質の標識において，注意する点について述べよ．

解　標識反応の反応条件や，標識部位によっては，タンパク質が変性し，その機能が失われることがある．

16.3　標識タンパク質を用いる分析法

16.3.1　タンパク質のアミノ酸配列決定

タンパク質の一次構造であるアミノ酸配列を決定することは，そのタンパク質の機能を調べるうえで欠かせないものである．エドマン(P. Edman)は一度に1個のアミノ酸残基を連続的に取り除く方法を開発し，これによりタンパク質のアミノ酸配列が決定できるようになった．このエドマン分解法とよばれる方法は，エドマン試薬として知られるフェニルイソチオシアネートを用いる．この方法により，最終的に生成したN末端アミノ酸のPTH誘導体は，紫外線を吸収するので，HPLCにより分離・同定できる．

16.3.2　タンパク質の構造変化の検出

細胞内におけるタンパク質は，つねに一定の構造をしているのではなく，低分子リガンドの結合やリン酸化などの刺激に応じて構造を変化させ，その機能を調節している．この構造変化を検出する方法として，蛍光共鳴エネルギー移動(fluorescence resonance energy transfer；FRET)や蛍光物質の環境に応じたスペクトル変化を利用した方法がある．前者はタンパク質を二種類の蛍光物質で標識し，この間で起こるFRETによるスペクトルの変化により構造変化を検出する(図16.5 a)．これに対して後者は，一種類の蛍光物質でタンパク質を標識する．タンパク質の構造変化が起これば，蛍光物質の環境(溶媒との相互作用，偏光などの物理化学的性質)が変化し，これがスペクトルの変化として検出される(図16.5 b)．これらの方法は，細胞の受容体やチャンネルのような膜タンパク質の機能解析に有効である．

蛍光共鳴エネルギー移動(fluorescence resonance energy transfer; FRET)は，ある蛍光物質(ドナー：D)が励起され，蛍光を発して基底状態に戻る際に，近傍に別の蛍光物質(アクセプター：A)が存在する場合，AにDの励起エネルギーが移動して，Aが蛍光を発するという現象である．ただしこれが起こるにはDの蛍光とAの吸収に波長の重なりがあることが必要である．

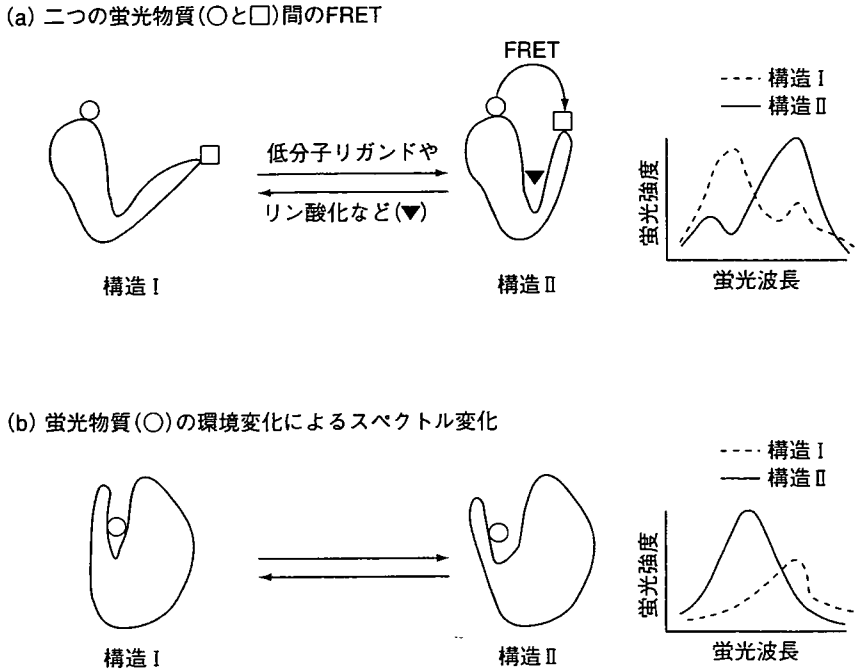


図 16.5　タンパク質の構造変化の検出

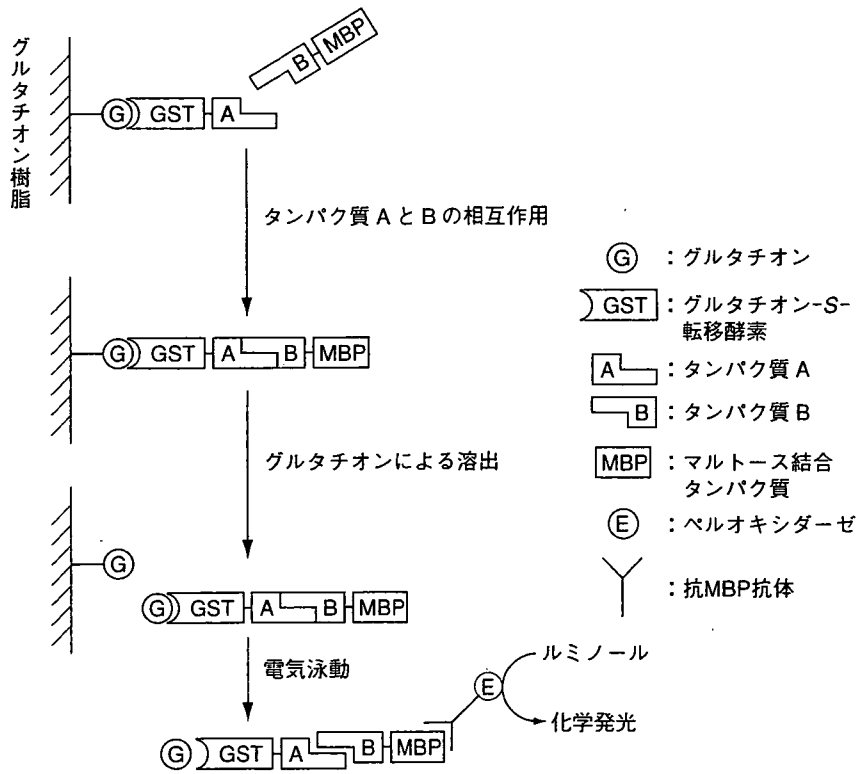


図 16.6　融合タンパク質を用いたタンパク質間相互作用による MBP タンパク質の化学発光検出

16.3.3　タンパク質間相互作用の評価

受容体，細胞内情報伝達，転写に関与するタンパク質などは，複数のタンパク質からなる複合体として機能していることが多い．このタンパク質間の相互作用(結合)を検出する方法として，融合タンパク質と標識抗体を利用したプルダウンアッセイ(pull down assay)がある．図16.6に示す方法では，タンパク質Aと Bの間で，特異的なタンパク質間相互作用が起これば，あとから加えた融合タンパク質のタグタンパク質(MBP)を特異的に認識できる酵素標識抗体と結合させることによって，タンパク質AおよびBとの相互作用やタンパク質Aの生成量を測定できる．

プルダウンアッセイ
タンパク質Aに対する抗体を用い，Aと相互作用するタンパク質を分離，同定する方法である．遺伝子発現実験などと組み合わせることで，未知のタンパク質の単離や新規のタンパク質間相互作用の有無を検出することができる．

16.4　核酸の標識

核酸の標識物質としては，^{32}P，^{35}Sなどの放射性同位元素が従来，一般的に用いられていたが，その使用や廃棄が厳しく制限されるので，現在，これにかわるものとしてフルオレセイン誘導体(FITC，FAM)，シアニン色素(Cy3，Cy5)，クマリン誘導体などの蛍光性の有機化合物を標識したヌクレオチドが開発されている(図16.7)．これらの標識ヌクレオチドは，DNAの塩基配列決定やハイブリダイゼーションに用いられる標識DNA(RNAまたはオリゴヌクレオチド)プローブの作製などに用いられる．

標識ヌクレオチドを用いた標識DNAの作製法としては，大きく分けて二つの方法がある．一つは，標識したいDNAの5′または3′末端を標識する末端標識(エンドラベル，end labeling)法であり，もう一つは，DNA中に，標識ヌクレオチドを取り込ませる鎖内標識(インターナルラベル，internal labeling)法である．末端標識法によってDNAの末端を標識する場合，DNA一分子に取り込まれる標識物質が一分子であるので，鎖内標識法に比べて感度は低い．しかし，一本鎖DNA，RNA，短いDNA断片，あるいは合成オリゴヌクレオチドの標識に，本法が多く適用される．鎖内標識法は，さまざまな方法が開発されており，なかでも，ニックトランスレーション(nick translation)法およびランダムプライマー伸長(random primer extension)法は，広く用いられている．

16.4.1　5′末端標識法(図16.8)

5′末端を標識する場合，T4ポリヌクレオチドキナーゼが必要である．この酵素は，DNAやRNAの5′末端にリン酸基を転移するので，^{32}Pで標識したATPなどを加えれば，5′末端を標識することができる．通常，アルカリホスファターゼで脱リン酸化したDNAに，T4ポリヌクレオチドキナーゼによるリン酸化反応で，[γ-^{32}P]dATPのγ位の^{32}Pを取り込ませる．したがって，この方法では，放射性同位元素(^{32}P)でしか標識できない．

16.4.2　3′末端標識法

3′末端の標識には，ターミナルデオキシヌクレオチジルトランスフェラーゼ