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Site-selective Post-translational Modification of Proteins Using an Unnatural Amino Acid, 3-Azidotyrosine

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An efficient method for site-selective modification of proteins using an unnatural amino acid, 3-azidotyrosine has been developed. This method utilizes the yeast amber suppressor tRNA^{Tyr}/mutated tyrosyl-tRNA synthetase pair as a carrier of 3-azidotyrosine in an *Escherichia coli* cell-free translation system, and triarylphosphine derivatives for specific modification of the azido group. Using rat calmodulin (CaM) as a model protein, we prepared several unnatural CaM molecules, each carrying an azidotyrosine at predetermined positions 72, 78, 80 or 100, respectively. Post-translational modification of these proteins with a conjugate compound of triarylphosphine and biotin produced site-selectively biotinylated CaM molecules. Reaction efficiency was similar among these proteins irrespective of the position of introduction, and site-specificity of biotinylation was confirmed using mass spectrometry. In addition, CBP-binding activity of the biotinylated CaMs was confirmed to be similar to that of wild-type CaM. This method is intrinsically versatile in that it should be easily applicable to introducing any other desirable compounds (*e.g.*, probes and cross-linkers) into selected sites of proteins as far as appropriate derivative compounds of triarylphosphine could be chemically synthesized. Elucidation of molecular mechanisms of protein functions and protein-to-protein networks will be greatly facilitated by making use of these site-selectively modified proteins.

Key words: azidotyrosine, modification, protein synthesis, suppression, tyrosyl-tRNA synthetase.

Abbreviations: aaRS, aminoacyl-tRNA synthetase; CaM, calmodulin; CBP-ECFPht, ECFP having N-terminal CaM-binding–peptide and C-terminal hexa-histidine; ECFP, enhanced cyan fluorescent protein, one of the enhanced green fluorescent protein colour variants; TyrRS, tyrosyl-tRNA synthetase; EC. 6. 1. 1. 1.; Y43G, yeast TyrRS with a replacement of tyrosine 43 by glycine.

Labelling of protein has been a powerful tool for investigation of protein folding, structure, function, interactions and localization. The generally used methods for protein labelling utilize endogenous thiol groups (cysteine) or amino groups (lysine), which can be specifically reacted with maleimide, etc. or succinimidyl ester, etc., respectively. However, these methods present a serious problem in that modifications occur at random sites since there are usually multiple target sites in a protein. To solve this problem, Yanagawa *et al*. reported a method for C-terminus-specific fluorescence labelling of proteins using puromycin derivatives (*1–3*). Puromycin is an analogue of the 3′ end region of Tyr-tRNA^{Tyr}, and causes halfway termination of translation by getting linked to growing polypeptide chains non-specifically. Yanagawa *et al*. turned this property into their advantage. Puromycin (or its derivatives) could be specifically attached to the C-terminus of a full-length protein if the translation reaction was carried out at a very low concentration of puromycin derivatives. This C-terminal labelling method is suited for quantitative analyses because the labelled protein contains only one fluorophore in the molecule. Although this method is unique in that unequivocal labelling of a protein is possible, it has an intrinsic defect that the site of modification is restricted only to the C-terminus of target proteins.

Recently, it has become possible to incorporate non-canonical (i.e. not specified in the genetic code) amino acids into proteins site-specifically in *in vitro* (*4–7*) and *in vivo* (*8–12*) translation systems. Schultz and co-workers (*13*) succeeded in site-specifically incorporating ketone functionality into proteins in an *Escherichia coli* cell-free translation system, and in modifying it chemoselectively with fluorescein hydrazide. Combining this strategy together with controlled post-translational modification methods has facilitated versatile labelling of proteins. However, the incorporation efficiency of this

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unnatural keto-amino acid into proteins was not so high, and was at the most 20–30% (*14*). In addition, although this modification must be carried out for a long period of time (36 h) under mild conditions, modification efficiency was at the most about 50%. As an alternative method, Hohsaka *et al*. (*15, 16*) reported that a fluorescent unnatural amino acid containing an anthraniloyl group could be incorporated into proteins using aminoacyl-tRNAs carrying a four-base anticodon in an *E. coli* cell-free translation system. This method was based on frameshift suppression, and had the advantage that multiple numbers of unnatural amino acids could be introduced into a single protein using different four-base codons. However, we cannot expect much for total yield of full-length target proteins using this method because it is limited by the amount of aminoacyl-tRNAs added into the reaction, which are 'chemically mis-acylated' and thus cannot be recycled.

We previously showed that the yeast suppressor $tRNA^{Tyr}_{(CUA)}$/tyrosyl-tRNA synthetase (TyrRS) pair could be utilized as a 'carrier' of extra amino acids in an *E. coli* translation system *in vivo* (*17*), and *in vitro* (*18*). Moreover, we reported that yeast TyrRS with the replacement of a tyrosine residue at position 43 by glycine (Y43G) was able to utilize several 3-substituted tyrosine analogues as substrates for aminoacylation (*19*). One of the promising substrate among these 3-substituted tyrosine analogues would be 3-azidotyrosine. The azido group reacts selectively and rapidly with phosphine in water at room temperature with a high yield and has been used for crosslinking studies (*20, 21*). Furthermore, Bertozzi and co-workers (*22, 23*) succeeded in azido-specifically labelling azido-sugars on the cell surface using triarylphosphine derivatives, and in incorporating azidohomoalanine into methionine sites of a protein in an *in vivo* translation system followed by its chemoselective modification with triarylphosphine reagents, although the modified sites were not restricted to a single specific site.

By taking advantage of this azido-specific modification reaction, we propose here an efficient and rapid method for site-selective biotinylation of proteins based on the site-selective introduction of 3-azidotyrosine in an *E. coli* cell-free translation system expanded with the yeast suppressor $tRNA^{Tyr}_{(CUA)}$/mutant TyrRS pair, followed by the modification of the azido group with triarylphosphine derivatives to incorporate a biotin group. The overall concept of site-selective post-translational modification of proteins using 3-azidotyrosine and triarylphosphine delivatives is schematically illustrated in Fig. 1.

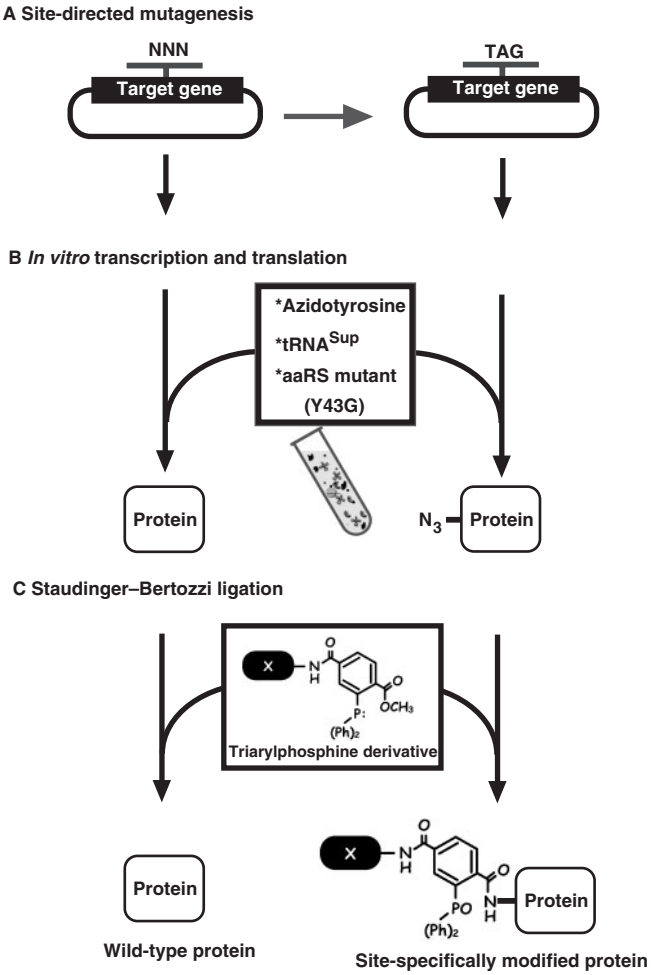

Fig. 1. **Schematic representation of the method for co-translational incorporation of 3-azidotyrosine into proteins at predetermined sites, and post-translational modification of the azido group using a triarylphosphine derivative.** (A) The desired position (NNN) in the target protein gene is mutated to an amber codon, TAG. (B) The target protein containing 3-azidotyrosine at the predetermined site is synthesized in an expanded *E. coli* transcription/translation coupled system. (C) The protein containing 3-azidotyrosine is chemoselectively labelled with a triarylphosphine derivative (X: desired residue, biotin in this case).

EXPERIMENTAL PROCEDURES

Materials—Non-radioactive L-amino acids were obtained from Peptide Institute Inc., and L-[^{35}S]-methionine was from Amersham Biosciences. Other chemical reagents for translation and mass spectrometry analysis were purchased from Roche Diagnostics Co., Sigma or Wako, Osaka, Japan. Reagents for detection of biotin, streptavidin, biotinyl alkaline phosphatase and the BCIP/NBT stable mixture were obtained from Sigma, Wako and Moss Inc., Maryland, USA, respectively.

Synthesis of 3-Azidotyrosine and Triarylphosphine–biotin Conjugate—3-Azido-L-tyrosine and 5-[3-diphenylphosphino-4-(methoxycarbonyl) benzamido] pentyl biotinamide (triarylphosphine-biotin conjugate, GIF-0470) were synthesized in our laboratory. Synthesis of 3-azidotyrosine has been described previously (*24*). As for the triarylphosphine–biotin conjugate, our preparation procedure was essentially based on that described by Kiick *et al*. (*22*) for triarylphosphine-FLAG. Analytical thin-layer chromatography (TLC) was performed on precoated (0.25 mm) silica gel plates (MERCK). ^{1}H and ^{13}C NMR spectra were obtained using a JEOL JNM AL-400 spectrometer with CD_3OD (CIL) as solvent. Chemical shifts (δ) are given in parts per million (ppm) downfield from a solvent as

internal reference with coupling constants (J) in Hz. Abbreviations s, d, t, q, m and br signify singlet, doublet, triplet, quartet, multiplet and broad, respectively. IR spectra were recorded using a SHIMADZU FTIR-8100A spectrophotometer with absorption bands given in cm^{-1}. Under Ar atmosphere, 5-(biotinamido)pentylamine (PIERCE) (48.0 mg, 146 mmol) was added to a solution of 2-(diphenylphosphanyl) terephthalic acid 1-methyl ester 4-(*N*-succinimidyl) ester (80.7 mg, 175 mmol) in DMF (1.5 ml) at room temperature, and the mixture was stirred for 20 h. To this mixture was added water, and the mixture was lyophilized to remove DMF. The crude product was purified by successive column chromatography using silica gel (MERCK) (CH_2Cl_2/$CH_3OH = 12/1$) and Sephadex LH-20 (CH_3OH) to give GIF-0470 (81.4 mg, 82.9%) as a yellow solid. TLC $R_f = 0.36$ ($CH_2Cl_2/CH_3OH = 9/1$); ^1H NMR (400 MHz, CD_3OD), δ 1.25–1.75 (m, 12H), 2.16 (t, 2H, $J = 7.2$ Hz), 2.68 (d, 1H, $J = 12.6$ Hz), 2.89 (dd, 1H, $J = 4.8$, 12.6 Hz), 3.11–3.20 (m, 3H), 3.26 (t, 2H, $J = 6.8$ Hz), 3.68 (s, 3H), 4.26 (dd, 1H, $J = 4.6$, 7.7 Hz), 4.46 (dd, 1H, $J = 4.8$, 7.7 Hz), 7.24–7.29 (m, 4H), 7.32–7.37 (m, 6H), 7.39 (dd, 1H, $J = 1.9$, 3.9 Hz), 7.76 (dd, 1H, $J = 1.9$, 8.2 Hz), 8.04 (dd, 1H, $J = 3.9$, 8.2 Hz), and four amide protons were not observed; ^{13}C NMR (100 MHz, CD_3OD), δ 25.2, 26.9, 29.5, 29.8, 29.9, 30.0, 36.8, 40.2, 40.8, 41.0, 52.6, 57.0, 61.6, 63.4, 127.7, 129.7 (4C, d, $J = 7.4$ Hz), 130.1 (2C), 131.6, 134.4, 135.1 (4C, d, $J = 21.3$ Hz), 138.1 (d, $J = 18.8$ Hz), 138.6 (2C, d, $J = 9.8$ Hz), 139.2, 142.4 (d, $J = 28.7$ Hz), 166.1, 168.4, 169.4, 176.0; IR (KBr, cm^{-1}) 536, 698, 747, 820, 858, 1026, 1057, 1117, 1161, 1190, 1254, 1281, 1298, 1435, 1455, 1462, 1480, 1505, 1538, 1565, 1644, 1651, 1667, 1682, 1694, 1711, 1723, 2859, 2930, 3076, 3286.

Non-radioisotopic Assay for Aminoacylation—The assay for aminoacylation or mis-aminiacylation was essentially based on that described by Ikeda *et al*. (*25*). The mutant TyrRS protein (Y43G) and *E. coli* prolyl-tRNA synthetase (ProRS) were prepared as described previously (*19, 26*). The suppressor $tRNA^{Tyr}_{(CUA)}$ was overproduced in *E. coli* BL21 (DE3) with the plasmid pGEMEX-supTyr harboring yeast amber suppressor $tRNA^{Tyr}_{(CUA)}$ (*27*), and purified by ion-exchange column chromatographies (*19*).

Aminoacylation mixture (10 μl total volume) contained 100 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 40 mM KCl, 4 mM ATP, 400 μM proline, 500 μM 3-azido-L-tyrosine, 0.1 A_{260}unit *E. coli* $tRNA^{mix}$, 0.1 μg *E. coli* ProRS, 1 μg Y43G and 0.01 A_{260}unit yeast suppressor $tRNA^{Tyr}_{(CUA)}$. The reaction was carried out at 30°C for 15 min and was stopped by lowering the pH to 4.75 with 6% acetic acid and aliquots were subjected to electrophoresis on 7 M Urea 15% polyacrylamide slab gel under acidic condition (pH 4.75). The separated RNA was transferred to a nylon membrane, crosslinked and hybridized with biotinylated probes 5′-CCC ATG ACG GTG CGC TAC CAG GCT biotin-3′ (for *E. coli* $tRNA^{Pro}$) or 5′-TCT CCC GGG GGC GAG TCG AAC GCC CGA TCT biotin-3′ (for yeast amber-suppressor $tRNA^{Tyr}$). The membrane was then subjected to detection for alkalinephosphatase-based chemiluminescence using Immun-Star™ AP substrates (BioRad). The RNAs remaining in the gel were stained with methylene blue.

Preparation of CaM Containing an Azido Group—The plasmid for wild-type (WT) calmodulin (CaM), pCaM-WT, was constructed as described previously (*28*), and substitution of positions 72, 78, 80 or 100 with the amber codon in the CaM gene was made using the QuikChange method (*29*). Primers used were 5′-TGA ATT CCT GAC ATA GAT GGC AAG AAA AAT-3′ (position 72A), 5′-ATT TTT CTT GCC ATC TAT GTC AGG AAT TCA-3′ (position 72B), 5′-GCA AGA AAA ATG TAG GAC ACA GAC AG-3′ (position 78A), 5′-CTG TCT GTG TCC TAC ATT TTT CTT GC-3′ (position 78B), 5′-AGA AAA ATG AAA GAC TAG GAC AGT GAA GA-3′ (position 80A), 5′-TCT TCA CTG TCC TAG TCT TTC ATT TTT CT-3′ (position 80B), 5′-GAT GGC AAT GGC TAG ATC AGT GCA GC-3′ (position 100A) and 5′-GCT GCA CTG ATC TAG CCA TTG CCA TC-3′ (position 100B), respectively.

The *E. coli* cell-free extract was prepared as described previously (*18*). The reaction mixture for transcription/translation coupled system (total volume, 50 μl) contained 50 mM Hepes-KOH (pH 7.5), 7.7 mM $Mg(OAc)_2$, 27.5 mM NH_4OAc, 200 mM KOAc, 1.7 mM DTT, 1.25 mM ATP, 0.83 mM GTP, UTP, CTP, 80 mM creatine phosphate, 0.21 mg/ml creatine kinase, 0.1 mg/ml T7 RNA polymerase, 4% polyethylene glycol (average molecular weight 8000), 3.4 A_{260}units/ml *E. coli* $tRNA^{mix}$, 30% *E. coli* cell extract, 80 μM tyrosine, 200 μM each 19 amino acids (except for tyrosine), 500 μM 3-azidotyrosine, 0.17 mg/ml TyrRS mutant (Y43G), 0.2 A_{260}unit/ml yeast suppressor $tRNA^{Tyr}_{(CUA)}$ and 0.02 mg/ml template DNA. After performing the reaction at 30°C for 1 h, 2.5 μl 1 M $CaCl_2$ were added to the mixture, and the translation products were purified using a column (25 μl) of Phenyl Sepharose CL-4B (Amersham Bioscience), eluting with 100 μl–100 mM Hepes–KOH (pH 7.5) and 1 mM EGTA (*28, 30*). In the experiment for suppression analysis, the reaction was performed in the presence of [^{35}S]-methionine, and the reaction mixtures were subjected to SDS–PAGE.

Labelling with Triarylphosphine Derivative and Detection of Labelled Proteins—In a typical labelling reaction, 100 μl of protein solution eluted from the Phenyl Sepharose CL-4B column was mixed with 5 μl 5 mM triarylphosphine–biotin conjugate in DMSO, and the mixture was incubated at 37°C for 30 min. Aliquots were withdrawn and subjected to SDS–PAGE for determination of the extent of biotinylation. Following separation of proteins by gel electrophoresis, a nitrocellulose membrane was placed onto the gel surface, and a stack of filter papers was placed on the membrane. Furthermore, a glass plate was placed on the top of papers and weighed down for 30 min. The membrane was blocked with a solution of 3%(w/v) BSA in TBS (10 mM Tris–HCl (pH 7.6) and 150 mM NaCl), and washed with TBST (10 mM Tris–HCl (pH 7.6), 500 mM NaCl and 0.05% Tween-20). Following treatment with a solution of 2.5 μg/ml streptavidin in TBST, the membrane was soaked in a solution of 0.1 μg/ml biotinyl alkaline phosphatase in TBST. Washes were repeated three times, and the membrane was subjected to colour development using BCIP/NBT substrates. Proteins remaining in the gel were stained with CBB.

Characterization of Labelled Proteins by Mass Spectrometry—Following the labelling reaction, proteins were precipitated with acetone. The collected proteins were mixed with 5 μl MALDI matrix [10 mg/ml 3,5-dimethoxy-4-hydroxycinnamic acid solution in 50% (v/v) acetonitrile and 0.05% TFA], and 1.5 μl of the mixture was applied onto a sample plate. Samples were analysed in the linear mode using an UltraFlex TOF/TOF mass spectrometer (Bruker Daltonics).

For the analysis of peptide mass fingerprinting and a mass/mass ion search method, labelling mixture were subjected to SDS–PAGE, and the separated protein bands were electrophoretically transferred to a PVDF membrane. Protein bands were visualized by staining with Ponceau S, and the band of interest was cut out. Digestion of proteins on the membrane was carried out essentially according to the method of Aebersold *et al*. (*31, 32*). The membrane was washed with 100 μl 50 mM NH_4HCO_3 in a microtube, and soaked in 10 mM dithiotreitol and 25 mM NH_4HCO_3. After 1 h, the solution was removed and replaced with 100 μl 55 mM iodoacetamide in 25 mM NH_4HCO_3. Alkylation was carried out at room temperature for 45 min in the dark. Then, the membrane was incubated with 20 μl 2.5 μg/ml trypsin in 25 mM NH_4HCO_3 at 37°C overnight, and the remaining liquid containing the resulting peptides was transferred to a new tube. One μl tryptic peptide solution was mixed with 4 μl MALDI matrix [saturated α–cyano-4-hydroxycinnamic acid solution in 33% (v/v) acetonitrile and 0.066% TFA], and 1 μl of the mixture was applied onto a sample plate. Samples were analysed in the reflector mode and LIFT method using a mass spectrometer.

Binding Activities of Biotin-labelled CaM—Biological activity of biotin-labelled CaM was assayed by formation of a complex with enhanced cyan fluorescent protein (ECFP) conjugated with CaM-binding peptide (CBP).

The plasmid pCAL-ECFPht was constructed by the following procedures. First, the coding region of ECFP in pECFP (Clontech) was amplified by PCR to create 5′ *Nco*I and 3′ *Xho*I sites, and was then inserted into pET29-a(+) (Novagen) for fusion. Primers used were: 5′-GGG GCC ATG GTG AGC AAG GGC GAG GAG CTG TT-3′ (primer A) and 5′-GGG GCT CGA GCT TGT ACA GCT CGT CCA TGC-3′ (primer B). Second, the coding region of the histidine-tagged ECFP was amplified by PCR to create 5′ *Nco*I and 3′ *Hin*dIII sites, and was inserted into pCAL-n (Stratagene) containing the CBP gene in the plasmid. Primer A and 5′-GGG GAA GCT TGC TTT GTT AGC AGC CGG ATC TCA-3′ (primer C) complementary to the neighbourhood of pET29-a(+) T7 terminator were used in this reaction. Expression was performed according to the manufacturer's protocols (Stratagene). Then, the histidine-tagged ECFP conjugated with CBP (CBP–ECFPht) was purified using columns of Ni-NTA agarose (1 ml, Qiagen) and Hi-Trap Q (1 ml, Amersham Biosciences). Concentration of the purified protein was determined by molar absorptivity. Rat CaM whose amino acid sequence is conserved completely among higher eukaryotes was purified as described previously (*28, 32*).

Reaction mixture for the complex formation (10 μl total volume) contained 100 mM Tris–HCl (pH 7.6), 1 mM $CaCl_2$, 8 mM CBP-ECFPht and 0.5 μg wild–type CaM or mutant CaM containing 3-azido–tyrosine. The reaction was carried out at 4°C for 4 h. Aliquots were subjected to electrophoresis on a 12.5% polyacrylamide slab gel containing 1 mM $CaCl_2$ for analysis of complex formation. The biotin-labelled protein bands were detected as described above and the protein bands remaining in the gel were visualized by staining with CBB.

RESULTS AND DISCUSSION

Site-selective Incorporation of 3-Azidotyrosine by the Expanded Cell-free Translation System—In order to establish the utility of the yeast suppressor $tRNA^{Tyr}_{(CUA)}$/Y43G pair as a 'carrier' of the extra amino acid, 3-azidotyrosine, in an *E. coli* translation system *in vitro* (*19*), it must be confirmed that the endogenous *E. coli* TyrRS does not use 3-azidotyrosine erroneously as substrate while retaining the ability to charge *E. coli* $tRNA^{Tyr}$ with tyrosine. Therefore, we first examined several reaction conditions for aminoacylation by *E. coli* TyrRS varying the L-[^{14}C]-tyrosine/3-azidotyrosine ratio. We found that *E. coli* TyrRS could not use 3-azidotyrosine at all as substrate in the presence of both 80 μM tyrosine and 500 μM 3-azidotyrosine (data not shown) and used these concentrations for both amino acids in the following experiments. Second, we examined if our mutant TyrRS, Y43G could mis-aminoacylate *E. coli* $tRNA^{Pro}$, since RajBhandary and co-workers had pointed out that wild-type yeast TyrRS had a tendency to misacylate *E. coli* $tRNA^{Pro}$ (*33*). Results of Acid PAGE assay for aminoacylation using Y43G and *E. coli* ProRS are shown in Fig. 2. The *E. coli* $tRNA^{Pro}$ bands were evidently delayed (aminoacylated) when *E. coli* ProRS was added in the reaction mixture (Fig. 2B, lanes 2, 4, 6 and 8). On the contrary, no delay of *E. coli* $tRNA^{Pro}$ was observed in the presence of Y43G (Fig. 2B, lanes 3 and 7), whereas the delay of suppressor $tRNA^{Tyr}$ was evident under the same condition (Fig. 2C, lanes 7 and 8). It was shown from this assay that Y43G could not mis-aminoacylate *E. coli* $tRNA^{Pro}$ with 3-azido-L-tyrosine, and the coexistence of Y43G did not influence prolylation of *E. coli* $tRNA^{Pro}$ by *E. coli* ProRS. This result agrees with our recent report (*19*).

CaM is one of the calcium-binding proteins and acts as a mediator of cellular Ca^{2+}-dependent signal pathways. Structure and function of CaM have been well investigated, and it can be easily synthesized in an *in vitro* translation system. In this study, we chose rat CaM (the sequence of which is conserved throughout the higher eukaryotes) as the model protein for incorporation of 3-azidotyrosine. The sites for incorporation of 3-azidotyrosine (positions 72, 78, 80 and 100) were determined by considering its NMR and X-ray crystallographic structures (*34, 35*). Figure 3 shows a typical example of *in vitro* CaM synthesis in the absence or presence of Y43G/3-azidotyrosine or suppressor $tRNA^{Tyr}_{(CUA)}$ using wild-type or mutant (80amb) genes for templates. As expected, introduction of the amber mutation at position 80 in CaM led to intra-peptide

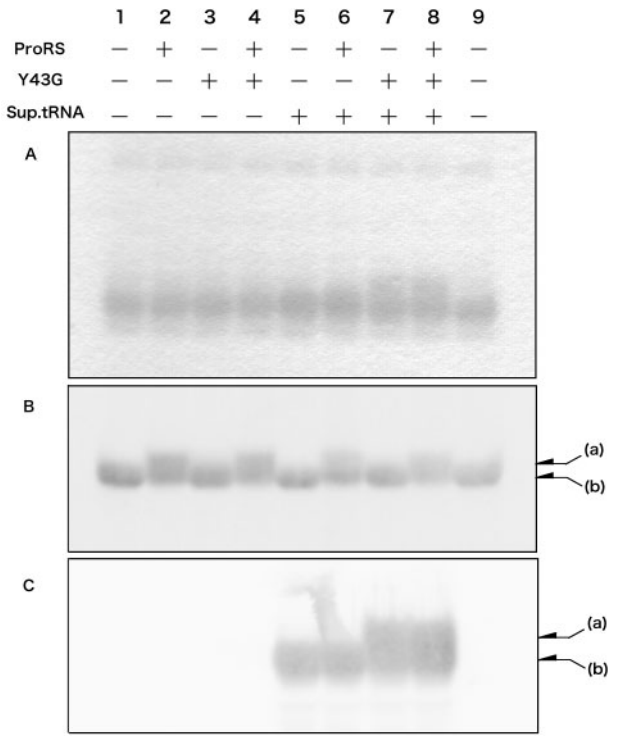

Fig. 2. **Aminoacylation of *E. coli* tRNA^{Pro} and yeast suppressor tRNA^{Tyr}.** Aminoacylation reactions with (+) or without (−) *E. coli* ProRS, Y43G or suppressor tRNA^{Tyr} were done in the presence of 0.1 A_{260}unit *E. coli* tRNA^{mix}, 400 μM proline and 500 μM 3-azidotyrosine at 30°C for 10 min. Aliquots of each reaction mixture were subjected to 7M urea Acid PAGE and the separated RNA bands were visualized either by staining with methylen blue (A), or by alkalinephosphatase-based chemiluminesence after blotting and hybridizing to each biotinylated DNA probe specific to *E. coli* tRNA^{Pro} (B), or to suppressor tRNA (C). The arrows indicate aminoacylated tRNAs (a) and non-acylated tRNAs (b).

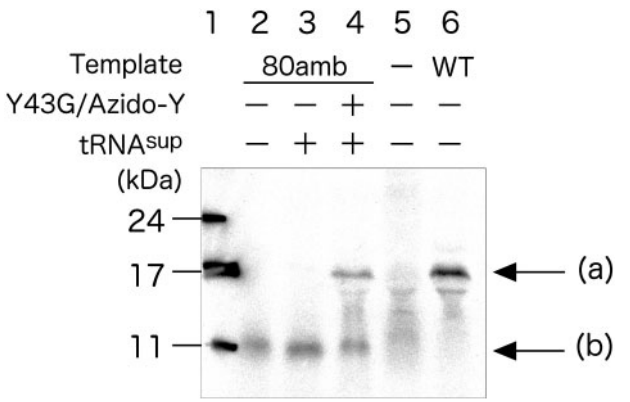

Fig. 3. **SDS–PAGE analysis of *in vitro* transcription/translation products of wild-type and mutant (80amb) CaM genes synthesized in the presence or absence of a yeast suppression system.** Transcription/translation reactions with (+) or without (-) Y43G/3-azidotyrosine or suppressor tRNA were done in the presence of [^{35}S]-methionine at 30°C for 1 h. The template used was rat CaM gene with a mutation from ACA80 to TAG80 (80amb, lanes 2–4) or without mutation (WT, lane 6). Aliquots of each reaction mixture were subjected to SDS–PAGE and the separated protein bands were analysed by BAS2500 (Fuji). Lane 5 is the result of the reaction in the absence of template DNA. The molecular-weight standards are in lane 1. The arrows indicate full-length CaM (read-through product) (a) and N-terminal peptide of CaM terminated at the amber codon 80 (b).

translation termination, producing a truncated peptide (Fig. 3, lane 2). The molecular mass of this peptide band (b) was estimated to be about 11 kDa, which was in good agreement with the value for the N-terminal 79 peptides (10.6 kDa) calculated from the reported nucleotide sequence of the rat CaM. Addition of the suppressor tRNA$^{Tyr}_{(CUA)}$ alone did not lead to read-through of the amber codon (Fig. 3, lane 3), indicating that the added yeast suppressor tRNA$^{Tyr}_{(CUA)}$ was not aminoacylated by any of the endogenous *E. coli* aaRSs at least under the conditions adopted in this experiment. On the other hand, full-length CaM was synthesized when suppressor tRNA$^{Tyr}_{(CUA)}$, Y43G and 3-azidotyrosine were added (Fig. 3, lane 4), and suppression efficiency was about 50% judging from the band intensities of the suppressed product (a) and the truncated peptide (b). Although we have recently reported the development of a mutant of yeast suppressor tRNA$^{Tyr}_{(CUA)}$ whose mis-acylation level by *E. coli* LysRS is greatly reduced (*18*), this 'improved' suppressor tRNA had not been used in this study because it is often possible to avoid such mis-acylation by properly adjusting the tRNA/aaRS ratio as the mis-acylation level itself is very low. However, we expect that the use of this tRNA (U4C/AS G-Cr mutant in (*18*) would lead to an improvement of 3-azidotyrosine incorporation. Mobility of the suppressed CaM (Fig. 3, lane 4a) was in good agreement with the wild-type gene products (Fig. 3, lane 6). We also tried to incorporate 3-azidotyrosine into the amber codon at position 72, 78 or 100 using the corresponding mutant genes. Basically, similar suppression patterns were observed in each case, and the efficiencies were almost the same (about 50%) regardless of the position of the amber mutation (data not shown).

Post-translational Modification Using Triarylphosphine Derivatives—As a first choice for post-translational modification of the incorporated azido group, we adopted the modified Staudinger ligation (Staudinger–Bertozzi ligation) reaction utilizing triarylphosphine derivatives, since the reaction between the azido group and the phosphine proceeds very specifically and rapidly in aqueous solution at a moderate temperature and with a high yield (*22, 23, 36, 37*). Moreover, this method has also an advantage that it would be easily applied to introducing any other compounds as far as we can chemically synthesize the desirable triarylphosphine derivatives. Actually, Tsao *et al*. (*37*) described a method for site-selective fluorescein-labelling of a protein (a streptavidin-binding peptide fused to the pIII protein of M13 phage) by incorporating *p*-azidophenylalanine into the target protein, followed by modification of its azido group by the Staudinger–Bertozzi ligation using a triarylphosphine–fluorescein conjugate.

In this article, we described site-selective biotinylation of higher eukaryotic CaM using 3-azidotyrosine and a triarylphosphine–biotin conjugate. Since biotin has the

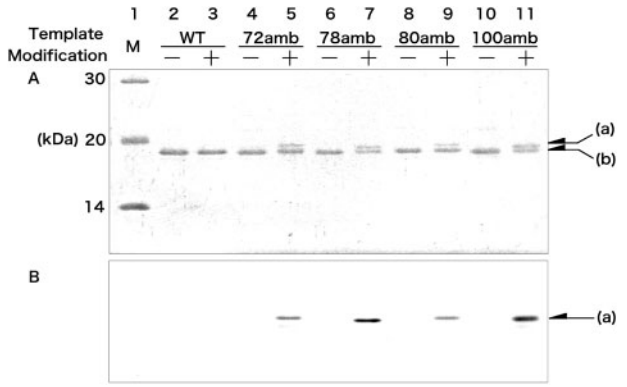

Fig. 4. **SDS–PAGE analysis of post-translational modification products of CaM.** Wild-type or amber-mutated (72, 78, 80 or 100 amb) genes were added to the transcription/translation mixture, and the reaction products were purified as described in experimental procedures. After the modification reaction with (+) or without (−) triarylphosphine–biotin conjugate at 37°C for 1 h, aliquots of each reaction mixture were subjected to SDS–PAGE. The separated proteins were partially transferred onto a nitrocellulose membrane as described in experimental procedures. The proteins remaining in the gel were visualized by CBB staining (A), and the blot membrane was treated for detection of biotin (B). The arrows indicate biotinylated CaM (a) and unmodified CaM (b).

ability to interact with avidin or streptavidin strongly and specifically (*38*), biotinylation of proteins has been widely used for detection, immobilization and capture of the interactive molecules (*39, 40*).

Results of post-translational modification with the triarylphosphine–biotin conjugate of wild-type CaM and its mutants containing 3-azidotyrosine are shown in Fig. 4. CaM is known to undergo large conformational change from the closed to open forms upon binding of Ca^{2+}. This Ca^{2+}-induced conformational change increases hydrophobicity of the CaM molecule. Therefore, hydrophobic chromatography is an effective method to prepare an active form of CaM (*25*). For this reason, we purified our *in vitro*-synthesized CaM mutants containing 3-azidotyrosine using a Phenyl-Sepharose CL-4B column in the presence of Ca^{2+}. Eluted proteins from the column by removing Ca^{2+} were analysed by SDS–PAGE (Fig. 4A, lanes 2, 4, 6, 8, and 10). Observations that intensities of the protein bands in these lanes were similar to that of wild-type CaM, and molecular masses of all the CaM preparations were apparently the same suggested that all the 3-azidotyrosine-containing CaM mutants had at least the ability to undergo this Ca^{2+}-induced conformational change.

As shown in Fig. 4B, the biotin-labelled CaM could be seen only in the sample originating from the amber-mutated CaM genes [bands (a)], indicating the site-selective incorporation of 3-azidotyrosine and azido-specific biotin-labelling. Modification efficiency was estimated to be about 30–50% judging from the intensities of the protein bands stained with CBB (Fig. 4A, lanes 5, 7, 9 and 11). Differences among the ratios of intensities between band (a)/band (b) at each site might reflect the accessibility of the azido grupe to the biotinylation reagent.

We analysed molecular mass of biotinylation products originating from a mutant gene (78amb) on a mass spectrometer. As shown in Fig. 5A, two major signals are observed. The peak (a) at $m/z = 17575.9$ Da corresponds to the expected mass of single-modified CaM (calc. 17575.45 Da) with substitution by 3-azidotyrosine for methionine and addition of triarylphosphine-biotin conjugate. Signals for multiple-modified CaMs are not seen in this spectrum. Another peak (b) at $m/z = 16917.7$ Da is nearly consistent with the value of supposed unmodified CaM having 3-amino-tyrosine at the amber-site (calc. 16916.70 Da). This 3-amino-tyrosine might have been generated by reduction of 3-azidotyrosine during the translation or modification reactions as Staros *et al.* (*41*) described that aryl azides were rapidly reduced to aryl amines depending on the reaction conditions used. If this is the case, the presence of 3-amino-tyrosine at the amber sites instead of 3-azidotyrosine would be a possible explanation for the incomplete biotinylation observed above.

To confirm 3-azidotyrosine incorporation and azido-specific biotinylation, and to determine its occupancy at the amber position (position 80 for example), mutant CaM was analysed by peptide mass fingerprinting and a mass/mass ion search method on a mass spectrometer. The precursor ion at $m/z = 2087.697$ Da (y11 in Fig. 5B) corresponded to the mass of tryptic peptide (MKDY*DSEEEIR) containing biotinylated 3-azidotyrosine (Y*) at position 80 (the lysine residue at position 78 was not cleaved). This precursor ion was selected and analysed using the mass/mass ion search method. The fragment masses could be assigned as shown in Fig. 5B. The partial sequence DY*DSEEEIR of the peptide containing the biotinylated 3-azidotyrosine residue (Y*) could be read from the chart of annotated y-ion series. Because the tryptic peptide sequence containing position 80 was MKDY*DSEEEIR, site-specific biotinylation was thus established. These results indicate that post-translational modification with triarylphosphine–biotin conjugate occurred specifically at the azido group incorporated at the amber-mutated site(s). The presence of presumed 3-amino-tyrosine at the amber site was also confirmed by detecting the corresponding fragment (data not shown).

Analysis of CBP-binding Activities of Biotin-labelled Calmodulin—Ability to form a complex between biotin-labelled CaM and CBP conjugated with his-tagged ECFP (CBP-ECFPht) was assessed by the gel-mobility-shift method. Figure 6 shows typical results of such binding analysis. A band-shift was observed when the *in vivo*-expressed CaM (lane 2, Ex CaM) or *in vitro*-synthesized wild-type gene product (lane 4, WT) was incubated with CBP-ECFPht (lanes 3 or 5, respectively). Therefore, the *in vitro*-synthesized CaM was shown to have similar binding activity to that of the *in vivo*-expressed CaM. As expected, azido-specifically biotinylated CaM (lane 6, Bio80) was also shifted similarly upon addition of CBP-ECFPht (lane 7). The result of biotin-detection confirmed that the biotinylated CaM did form a complex with CBP-ECFPht (Fig. 6B, lane 7). Other samples of

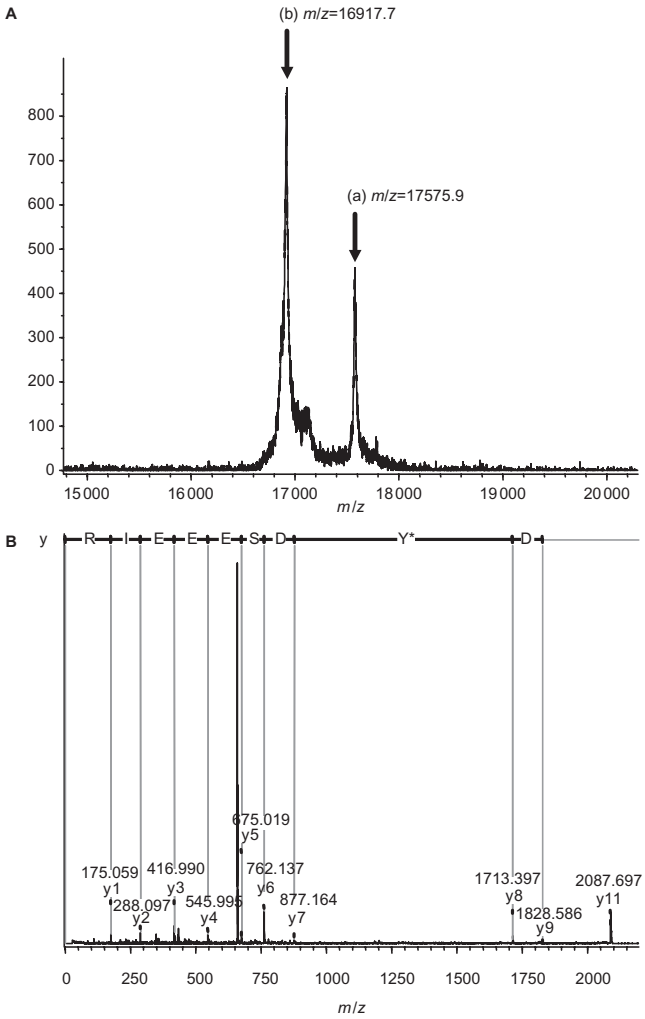

Fig. 5. **MALDI-mass spectra of the modification products and the biotinylated fragment.** (A) MALDI-mass spectrum of biotinylated CaM and unlabelled CaMs. The biotinylated CaM molecule was prepared as described in experimental procedures. Following the precipitation by acetone, the MALDI matrix was added. The samples were analysed using the UltraFlex TOF/TOF mass spectrometer (Bruker Daltonics). The arrows indicate signals at $m/z = 17575.9$ Da (a) and $m/z = 16917.7$ Da (b). (B) Tandem mass spectrum of the biotinylated fragment. The biotinylated CaM molecule was separated from unlabelled CaMs by SDS–PAGE, and transferred electrically onto a PVDF membrane. The protein band of interest on the membrane was cut out and treated by reduction and alkylation, followed by incubation with trypsin overnight at 37°C, as described. The MALDI matrix was added, and samples were analysed using a mass spectrometer. The precursor ion at $m/z = 2087.697$ Da was selected, and analysed by mass/mass ion search method. Y* indicates the biotinylated 3-azidotyrosine residue.

CaM biotinylated at positions 72, 78 or 100 also showed similar abilities to form a complex with CBP-ECFPht (data not shown).

These results demonstrated that our method for site-selective biotinylation of proteins based on the co-translational introduction of 3-azidotyrosine and post-translational modification of the azido group with

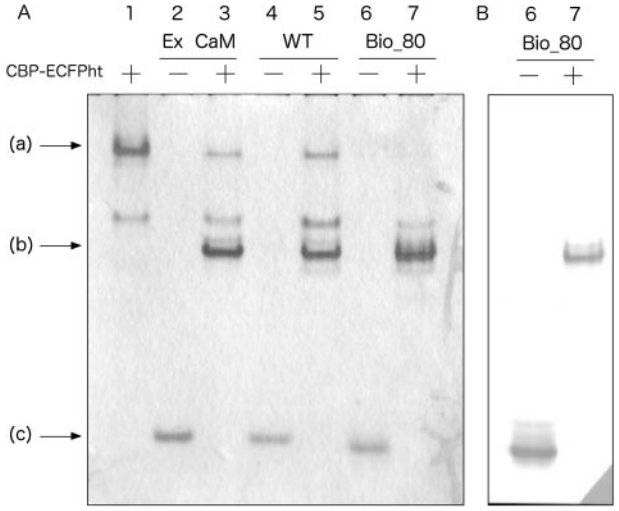

Fig. 6. **Analysis of substrate-binding activity of CaM.** *In vitro* transcription/translation products of wild-type and mutant (80amb) CaM genes were treated with triarylphosphine–biotin conjugate (WT and Bio_80, respectively). For removing unreacted triarylphosphine–biotin conjugate, reaction products were purified using Phenyl Sepharose CL-4B columns. Binding reaction was done at 4°C for 4 h in the absence (−) or presence (+) of 8 mM CBP-ECFPht. Aliquots of the reaction mixture were applied onto a 10% polyacrylamide slab gel containing 1 mM $CaCl_2$. After electrophoresis, separated proteins were visualized by CBB staining (A) or by biotin detection (B). The arrows indicate CBP-ECFPht (a), complex of CBP-ECFPht and (biotinylated) CaM (b) and CaM or biotinylated CaM (c); Ex CaM represents *in vivo*-expressed wild-type CaM.

triarylphosphine derivatives is easy, efficient and rapid. This biotinylation system will be applicable to investigations of protein-to-protein or protein-to-ligand interactions in combination with streptavidin magnetic beads. In addition, it should be noted that this method is easily applicable to introducing any other desirable compounds into selected sites of proteins as far as appropriate triarylphosphine derivatives could be chemically synthesized. Site-selective fluorescence-labelling or PEGylation of proteins by using triarylphosphine–fluorophore conjugate or triarylphosphine–polyethylene glycol conjugate is now underway in our laboratory. On the other hand, we noticed that the overall modification efficiency was typically in the range of about 30–50%, not so high as we had expected. One possible explanation for this incomplete modification would be the reduction of 3-azidotyrosine to 3-amino-tyrosine during the course of reaction. Establishing the optimal reaction conditions to minimize such reduction reaction would be of much help to improve the overall modification efficiency.

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