A non-natural amino acid for efficient incorporation into proteins as a sensitive fluorescent probe

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Abstract A small and highly fluorescent non-natural amino acid that contains an anthraniloyl group (atnDap) was incorporated into various positions of streptavidin. The positions were directed by a CGGG/CCCG four-base codon/anticodon pair. The non-natural mutants were obtained in excellent yields and some of them retained strong biotin-binding activity. The fluorescence wavelength as well as the intensity of the anthraniloyl group at position 120 were sensitive to biotin binding. These unique properties indicate that the atnDap is the most suitable non-natural amino acid for a position-specific fluorescent labeling of proteins that is highly sensitive to microenvironmental changes.

Key words: Non-natural mutagenesis; Fluorescent amino acid; In vitro protein synthesis; Streptavidin

1. Introduction

Position-specific incorporation of non-natural amino acids into proteins through an in vitro biosynthesizing system is a versatile technique in biochemistry [1–4]. We have extended the technique by incorporating a variety of non-natural amino acids by using four-base codon/anticodon pairs [5–8]. One of the potential applications of non-natural mutagenesis is a fluorescent labeling of proteins at specific positions [9,10]. By incorporating proper fluorescent probes at appropriate positions into receptors, antibodies, and enzymes, we can detect ligands, antigens, and inhibitors sensitively without suppressing the original binding activity. For the fluorescent probe to work effectively, however, the fluorescent group must be highly sensitive to a small change of microenvironment caused by the binding of small molecules. Moreover, the position of the probe must be optimized to observe changes in the fluorescence intensity or wavelength with ligand binding, but not to suppress the binding itself [9].

However, incorporation efficiencies of amino acids carrying large fluorophores such as dansyl [3,10] and pyrenyl groups [7] are usually very low, mainly due to their bulky side groups. Therefore, it is essential to find small and sensitive fluorescent amino acids that are efficiently incorporated into proteins. To this aim, we designed two fluorescent amino acids that contain anthraniloyl groups. The anthraniloyl group is a small fluorescent chromophore compared to most of the fluorophores commonly used for protein labeling [11,12] and will give rise to minimum conformational perturbations when they are incorporated into proteins. Moreover, the anthraniloyl group emits fluorescence at wavelengths longer than 400 nm and can be detected without interference from the intrinsic tryptophan fluorescence. Incorporation of β-anthraniloyl-L-α,β-diaminopropionic acid (atnDap) into proteins has been referred to by Ellman et al. [1], but details of the synthesis, incorporation efficiency and spectroscopic data have not been reported.

In the present paper, the synthesis and fluorescence properties of streptavidin incorporated with a single atnDap unit at a specific position are described.

2. Materials and methods

2.1. Instruments

MALDI-TOF mass spectra were taken on a Voyager DE Pro instrument. Sinapinic acid (for protein measurements) and 2,5-dihydroxybenzoic acid (for other organic compounds) were used as matrices with concentration 10 mg/ml in acetonitrile/water (1:1, v/v). NMR spectra were recorded on a Varian Mercury 300 spectrometer. Each reaction was monitored by analytical reverse-phase HPLC (Waters, μBondasphere, 5μC18 100A, 3.9×150), flow rate 0.6 ml/min with gradient 0–100% methanol in 0.1 M ammonium acetate (pH 4.5) over 50 min.

2.2. Synthesis of the amino acid and aminoacylation of pdCpA (*4a*)

To a stirring solution of $^{\alpha}N$-Boc-L-α,β-diaminopropionic acid **1** (25.0 mg, 122 μmol) and Na_2CO_3 (12.9 mg, 122 μmol) in water/acetonitrile (1:1 v/v, 1.2 ml), isatoic anhydride **2a** (23.8 mg, 146 μmol) in acetonitrile was added dropwise at ice temperature [13]. The mixture was stirred overnight under a nitrogen atmosphere initially at 0°C and then at room temperature. The mixture was acidified to pH 2 with 5% aqueous $KHSO_4$ and extracted with ethyl acetate. The extract was washed with saturated aqueous NaCl, dried over sodium sulfate, and concentrated under reduced pressure to give 36.5 mg (93% yield) of $^{\alpha}N$-Boc-β-anthraniloyl-L-α,β-diaminopropionic acid (Boc-atnDap) **3a**, which was subjected to the next reaction without further purification.

3a: ^{1}H NMR ($CDCl_3$) δ 7.45–7.20 (2H, m), 7.21 (1H, s, broad), 6.79–6.65 (2H, m), 6.12 (1H, s, broad), 4.43 (1H, m), 4.04–3.63 (4H, m), 1.50 (9H, s); MALDI-TOF MS (*m*/*z*) 325.2 $[M+H]^+$, 347.2 $[M+Na]^+$.

Triethylamine (9.1 μl) and **3a** (3.1 mg, 9.6 μmol) were mixed in acetonitrile (310 μl) at ice temperature and then chloroacetonitrile (3.1 μl) was gradually added. The mixture was stirred overnight and

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brought to room temperature. It was acidified to pH 2 with 5% aqueous $KHSO_4$, and extracted with ethyl acetate. The extract was washed with 4% aqueous $NaHCO_3$, saturated aqueous NaCl, and dried over sodium sulfate. It was concentrated under reduced pressure to give 3.3 mg (95% yield) of **5a** as a yellow solid, which was subjected to the next reaction without further purification.

5a: HPLC $R_f = 36$ min; ^1H NMR ($CDCl_3$) δ 7.39–7.22 (2H, m), 6.77–6.65 (2H, m), 6.59 (1H, m), 5.71 (1H, m), 5.51 (1H, s, broad), 4.87 (1H, s), 4.58 (1H, m), 3.99–3.81 (2H, m), 1.51 (9H, s); MALDI-TOF MS (*m/z*) 364.2 $[M+H]^+$, 386.3 $[M+Na]^+$.

The aminoacylation of pdCpA was carried out by adding excess cyanomethyl ester **5a** to a dry DMF solution of pdCpA tetra-*n*-butylammonium salt in a microtube. The resulting solution was incubated at 37°C overnight and the reaction was monitored by reverse-phase HPLC. After the disappearance of pdCpA, 1 ml of diethyl ether was added to the solution and the resulting precipitate was collected by a centrifuge. The precipitate was dissolved in a small amount of acetonitrile and then precipitated again by adding 1 ml of ether. The washing was repeated twice to remove unreacted cyanomethyl ester. The pellet was dried and analyzed by HPLC ($R_f = 31$ min).

The Boc group was removed by treatment with trifluoroacetic acid (TFA, 200 μl) at 0°C for 10 min. After evaporation of excess TFA, the pellet was washed twice with diethyl ether. It was dried in vacuo to give the desired aminoacylated pdCpA **4a** in almost quantitative yield.

4a: HPLC $R_f = 24$ min; MALDI-TOF MS (*m/z*) 843.0 $[M+H]^+$, 865.1 $[M+Na]^+$, 887.1 $[M+2Na]^+$, 909.1 $[M+3Na]^+$.

2.3. In vitro protein biosynthesis

Protein biosynthesis was carried out as follows [6]. The aminoacylated pdCpA **4a** was coupled with the $tRNA_{CCCG}$(-CA) with T4 RNA ligase. The full-length aminoacylated tRNA with a CCCG four-base anticodon was mixed with the in vitro biosynthesizing system of *Escherichia coli* S30 lysate (Promega) together with the mRNA for mutant streptavidin [9]. The synthesis of the full-length mutant streptavidins was confirmed both on SDS–PAGE followed by Western blot analysis using anti-T7 tag antibody (Novagen) and alkaline phosphatase-labeled anti-mouse IgG (Promega) and by MALDI-TOF mass spectroscopy. For the mass spectroscopic studies, the reaction mixture (100 μl) was centrifuged at 15 000 rpm for 10 min, then the supernatant was loaded onto 10 μl of a TALON column (Clontech) equilibrated with buffer A containing 50 mM sodium phosphate and 300 mM NaCl at pH 7.0. The column was washed with seven 1 ml portions of buffer B that contained 50 mM sodium phosphate, 1 M NaCl, and 5 mM imidazole (pH 7.0), and then with two 0.2 ml portions of buffer A. The protein was eluted with 20 μl of elution buffer that contained 50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, and 0.05% PEG8000 at pH 7.0. The purified protein was desalted and concentrated by using $ZipTip_{C18}$ silica resin (Millipore) according to the given procedure, and eluted directly onto the MALDI target.

The binding activity of the mutant streptavidins against biotin was tested for the in vitro reaction mixture by a dot blot analysis using biotin-linked alkaline phosphatase (Zymed).

Mutant streptavidin that contained β-(*N*-methylanthraniloyl)-L-α,β-diaminopropionic acid (nmaDap) was also synthesized in a similar manner.

2.4. Fluorescence measurement

Fluorescence spectra were measured as previously described [9]. The reaction mixture (100 μl) was purified by the TALON column as described above. The eluate (70 μl) was diluted by four portions of buffer A and transferred into a microcuvette under argon. Fluorescence spectra were recorded on a Spex-Joan-Yvon Fluoromax2. The slit width was 1.5 nm for excitation and 5 nm for emission.

3. Results and discussion

3.1. Synthesis, characterization and biotin-binding activities of mutant streptavidins

The key intermediates (aminoacyl pdCpAs) **4** containing the anthraniloyl groups were easily synthesized in high yields from commercially available reagents as shown in Fig. 1.

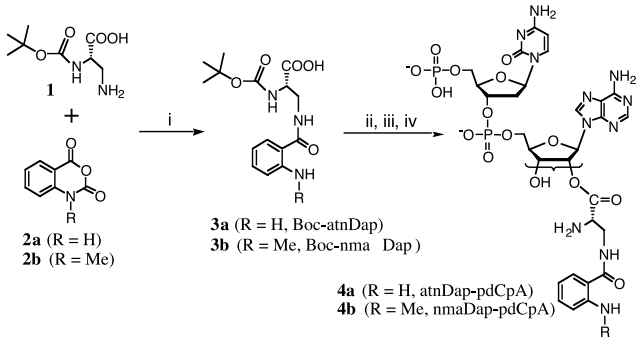

Fig. 1. Reagents and conditions: i, Na_2CO_3, $MeCN/H_2O$, 0°C to room temperature, 93%; ii, triethylamine, chloroacetonitrile/MeCN, 0°C to room temperature, 95%; iii, pdCpA, dry DMF, 37°C, quantitative; iv, CF_3COOH, 0°C, quantitative.

tRNAs containing four-base anticodons and charged with the respective fluorescent amino acids were prepared according to the established method [6]. Mutant streptavidin genes containing a CGGG codon at various sites were generated by the PCR method and the corresponding mRNAs were prepared by T7 RNA polymerase [9]. Then the tRNAs and mRNAs were added into the *E. coli* in vitro protein biosynthesizing system [6]. The mutated streptavidins were confirmed by Western blotting using anti-T7 tag antibody and alkaline phosphatase-labeled anti-mouse IgG (Fig. 2a), by MALDI-TOF mass spectroscopy, and by dot blotting using biotin-labeled alkaline phosphatase (Fig. 2b). The Western blots indicate that full-length streptavidins were produced in all cases. Densitometric analysis of the Western blots indicated that the yields of the mutant streptavidins were around 50% of the wild-type with a variation depending on the incorporation positions. TOF mass spectroscopy showed corresponding peaks for the wild-type and mutant streptavidins. Wild-type: (*m/z*) found 18902, calcd. for $[M+H]^+$: 18898; Ser52atnDap: (*m/z*) found: 19024, calcd. for $[M+H]^+$: 19016; Arg84atnDap: (*m/z*) found: 18957, calcd. for $[M+H]^+$: 18947.

The biotin-binding activities strongly depended on the positions of the incorporation. They were lost when the atnDap was incorporated into the inner hydrophobic region (at positions 21, 75, 79, 80, 92, and 108). Some of the mutants, such as Arg84atnDap and Trp120atnDap, were synthesized in excellent yield (over 70% of the wild-type) and also retained strong biotin-binding activity.

In contrast to the atnDap case, the incorporation efficiency of nmaDap was very poor (~10% compared to the wild-type). Moreover, the 120nmaDap streptavidin showed no biotin-binding activity. Steric repulsion by the *N*-methyl group seems to disrupt the protein conformation.

3.2. Fluorescence properties of Trp120atnDap mutant streptavidin

The Trp120atnDap mutant, which retained strong biotin-binding activity, was purified with an affinity column for the histidine tag [9]. Fluorescence spectra of the purified protein were measured in the presence of various amounts of biotin (Fig. 3). The peak gradually shifted to shorter wavelengths and the fluorescence intensity around 400 nm increased with the binding of biotin, indicating microenvironmental changes around position 120 [14]. It is noteworthy that the binding

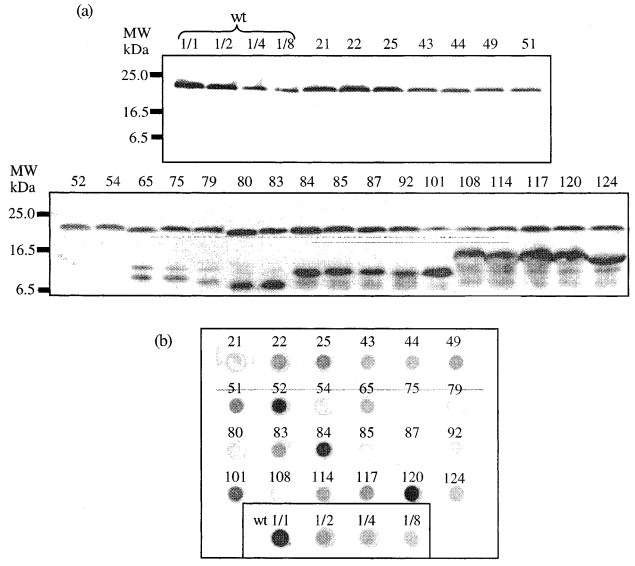

Fig. 2. Western (a) and dot blot (b) analysis of the translation products of wild-type and atnDap streptavidins prepared in the S30 in vitro biosynthesizing system. The numbers indicate the positions of atnDap units. For a rough estimate of the incorporation efficiency, Western blots were made for the wild-type protein at different dilution factors. No mutant was formed in the absence of atnDap-tRNA$_{CCCG}$.

causes a shift of fluorescence wavelength as well as an intensity change. Because the fluorescence wavelength does not depend on the probe concentration, it is a more convenient and reliable measure for microenvironmental changes. In this respect, the atnDap unit works as a better fluorescent probe than other fluorescent amino acids including those previously reported from our laboratory [9].

The inset of Fig. 3 shows the fluorescence intensity at 400 nm and the wavelength plotted against the biotin concentration. Least-squares analysis of the intensity change gave a

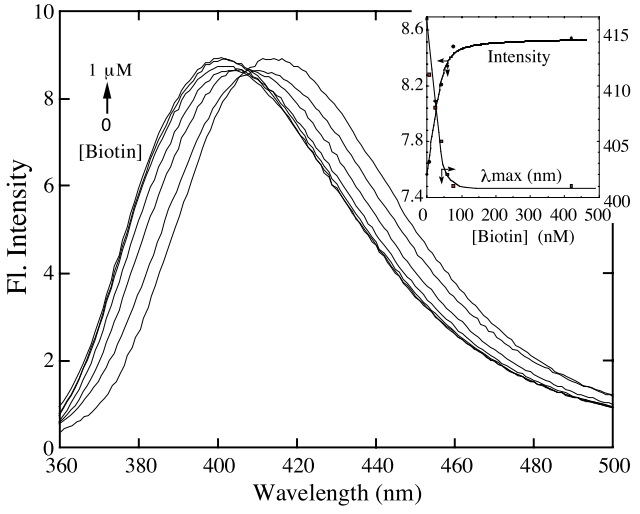

Fig. 3. Fluorescence spectra of 120atnDap streptavidin in the presence of different amounts of biotin in phosphate buffer (50 mM sodium phosphate and 300 mM NaCl at pH 7.0). λ_{ex} = 343 nm. The inset shows the fluorescence titration curves for the intensity and the wavelength against biotin.

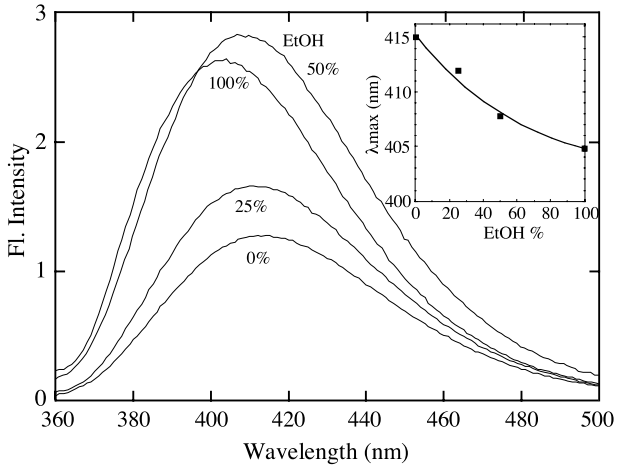

Fig. 4. Fluorescence spectra of atnDap in ethanol/phosphate buffer (50 mM sodium phosphate and 300 mM NaCl at pH 7.0) mixtures of different ratios. [atnDap] = 0.4 μmol/l, λ_{ex} = 330 nm.

binding constant $K = 2.0 \times 10^8$ M^{-1}, which is sufficiently large for the detection of nanomolar concentrations of biotin.

In contrast to the 120atnDap mutant, the fluorescence wavelength and intensity of purified 84atnDap mutant were insensitive to biotin binding, indicating the microenvironment of position 84 is not affected by biotin binding. This result suggests that the position of the fluorescent probe is another important factor for realizing sensitive detection by fluorescent-labeled proteins. The position-specific incorporation of fluorescent non-natural amino acids is the most promising technique to achieve this goal.

Fig. 4 shows fluorescence spectra of the amino acid atnDap in ethanol/phosphate buffer mixtures of different ratios. The peak position shifted to shorter wavelengths continuously from 0% ethanol (415 nm) to 100% ethanol (403 nm). A similar tendency has been reported previously [12]. Thus, the blue shift of the fluorescence of the 120atnDap mutant from 416 nm to 401 nm by biotin binding may be reasonably interpreted in terms of the relocation of the anthraniloyl chromophore from the hydrophilic region to a hydrophobic region. The relocation of the chromophore incorporated at position 120 has also been postulated for other fluorescent mutants [9].

3.3. Conclusions

The fluorescent amino acid atnDap could be synthesized easily and efficiently incorporated at several positions of streptavidin. Most of the mutants retain the biotin-binding activity. The fluorescence wavelength as well as the intensity of the 120atnDap mutant were sensitive to the binding. Because of the sensitivity of its fluorescence wavelength to the microenvironment and the compactness of the chromophoric side group, the atnDap unit is the most suitable fluorescent probe that can be incorporated at specific positions of proteins.

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