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Position-specific incorporation of a highly photodurable and blue-laser excitable fluorescent amino acid into proteins for fluorescence sensing

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Abstract—A new fluorescent amino acid, L-2-acridonylalanine, was incorporated into proteins at specific positions using 4-base codon/anticodon strategy. The efficiency of the incorporation was high enough to obtain enough quantities of the mutants. The acridonyl group was highly fluorescent when it was excited at the wavelengths of blue-lasers and was highly photodurable compared with conventional fluorophores often used for biological analyses. The fluorescence intensity was sensitive to small changes in the polarity of the environment. When the nonnatural amino acid was incorporated into specific positions of streptavidin, the mutant protein worked as a fluorescent sensor to biotin. Similarly, when the amino acid was incorporated into camel single-chain antibody, the mutant protein sensitively responded to the antigen molecule. The high incorporation efficiency, the high photodurability, the excitability with blue-lasers, and high sensitivity to the environment make the acridonylalanine as the promising fluorescent amino acid for sensing small molecules when incorporated into specific positions of various antibodies, receptors, and enzymes.

1. Introduction

Position-specific incorporation of nonnatural amino acids is a powerful tool for introducing speciality functions to various proteins.[1,2] Among a variety of possible applications, position-specific fluorescence labelling is promising as tools for wide variety of biochemical studies including proteome analysis.[3–5] For the position-specific fluorescence labelling to be really practical, however, development of appropriate fluorescent amino acids is essential. The types of fluorescent amino acids so far introduced into proteins by the ribosome-mediated nonnatural mutagenesis are still limited,[6–10] although wider range of fluorescent amino acids have been used in peptide synthesis.[11,12] Besides high incorporation efficiency into proteins,[13] the amino acid must show high fluorescence quantum yield, high durability to prolonged photoirradiation and proper excitation and emission wavelengths. The fluorescence intensity may be sensitive to the polarity of the surrounding microenvironment for sensitive detection of small molecules. As to the excitation wavelengths, recent development of fluorescence image analyzers and confocal microscopes equipped with blue-lasers prompted us to develop fluorescent amino acids that are excitable at the wavelength between 400 and 420 nm.

In this article, we present a new fluorescent amino acid that fulfills all the above requirements. L-2-Acrydonylalanine (I)[14] can be efficiently incorporated into proteins through 4-base codon/anticodon strategy,[1–5] shows a high quantum yield in polar solvents and a moderate quantum yield in nonpolar media, is highly durable to prolonged irradiation and shows absorption maximum

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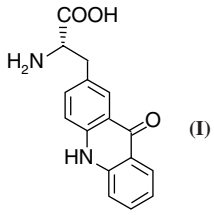
at the blue-laser wavelength, 405 nm. The amino acid was incorporated into various positions of streptavidin or camel single-chain antibody, cAb, against lysozyme. The mutant proteins were found to work as fluorescence sensors for biotin and antigenic lysozyme.



(I)

2. Results and discussion

2.1. Incorporation efficiency through *E. coli* S30 in vitro protein synthesizing system

Western blotting profiles for mutants of streptavidin and cAb are shown in Figure 1a and b, respectively. Yields of the nonnatural mutants of streptavidin were 40–71% depending on the mutation positions and 52–56% for cAb mutants, with respect to the wild-type protein. The incorporation efficiency into streptavidin is higher than those of other fluorescent amino acids; γ-(7-methoxycoumarin-4-yl)methyl-L-homoalanine (13%),[3] 2-anthrylalanine (40%),[3] and 2,6-dansylaminophenylalanine (17%).[5] As we have reported previously,[13] nonnatural amino acids with linearly extended side groups often show high incorporation efficiency and acdAla is a typical amino acid that follows this rule.

2.2. Photodurability of acridonyl group

Photodurability of acridonyl fluorophore was compared with those of other fluorescent groups frequently used in biochemical experiments. Figure 2 compares rates of the photobleaching of various fluorophores when they were continuously irradiated at their absorption maxima under aerobic conditions. Acridonyl group retained more than 95% of its original fluorescence intensity after 1 h irradiation at 385 nm, whereas fluorescence intensities of BODIPY and fluorescein decreased more rapidly under irradiation. These data indicate that acridonyl group is a tough enough fluorophore for prolonged measurement on fluorescence imagers or confocal microscopes equipped with blue-lasers.

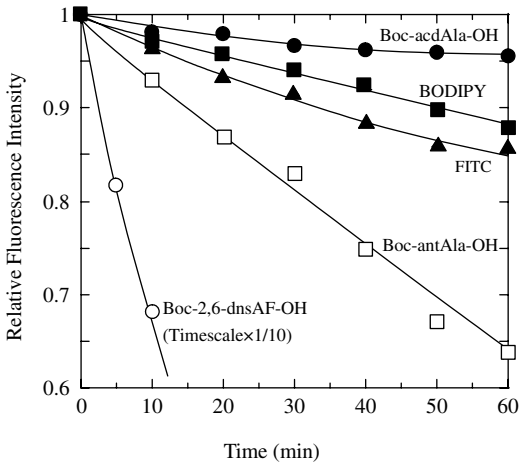


Figure 2. Photobleaching of various fluorophores under continuous irradiation at each absorption maximum in air-equilibrated aqueous solution. Absorbances at the absorption maxima were adjusted to be 0.1. The fluorescent compounds and their absorption maxima are: Boc-acdAla-OH, N-*t*-butyloxycarbonyl-2-acridonylalanine (385 nm), BODIPY, 4-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propanoyl)-aminophenylalanine (504 nm), FITC, fluorescein 5-isothiocyanate (490 nm), Boc-antAla-OH, N-*t*-butyloxycarbonyl-2-anthrylalanine (340 nm),[3] Boc-2,6-dnsAF-OH, N-*t*-butyloxycarbonyl-4-(2,6-dansylamino)phenylalanine (330 nm).[5] The bleaching rate of the last compound was so fast that the time scale was expanded by a factor of 10.

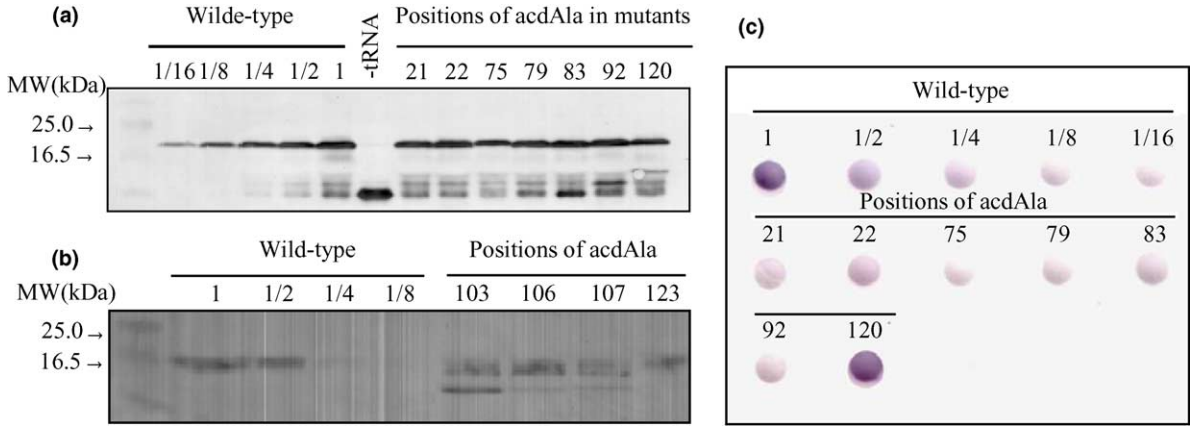


Figure 1. (a) Yields of acdAla mutants of streptavidin from an *E. coli* S30 in vitro system on Western blotting using anti-T7 tag antibody. The incorporation efficiency was evaluated by comparing band intensities of the mutant streptavidins with serially diluted wild-type streptavidin. (b) Yields of acdAla mutants of camel single-chain antibody. (c) Biotin binding activity of acdAla mutants of streptavidin evaluated by dot blot analysis using biotinylated alkaliphosphatase.

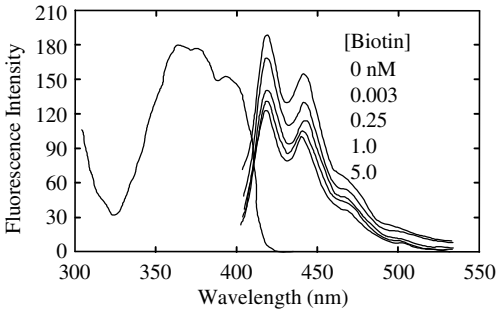


Figure 3. Fluorescence and fluorescence excitation spectra of ^{83}acdAla-streptavidin in 50 mM $NaHPO_4$, 300 mM NaCl solution containing 0.02% polyethyleneglycol at pH = 7.0, 25 °C, in the presence of various amounts of biotin. The excitation wavelength was 385 nm for fluorescence spectra and the fluorescence wavelength was 445 nm for excitation spectrum.

2.3. Fluorescence sensing of biotin with acdAla-streptavidins

Fluorescence and fluorescence excitation spectra of ^{83}acdAla-streptavidin in the presence of various amounts of biotin are shown in Figure 3.

Fluorescence of acridonyl group was quenched with the addition of biotin. The fluorescence peak intensity was plotted against biotin concentration in Figure 4. In the latter figure, fluorescence titration curves for other mutants that contained acdAla unit at different positions are also shown. The quenching efficiency largely depended on the positions of acdAla unit. For example, both the ^{83}acdAla and ^{120}acdAla mutants showed sharp changes in the titration curves indicating that the two mutants retained strong biotin-binding activity and the bound biotin caused decrease of the acridonyl fluorescence, probably by the decrease of polarity, as will be discussed below. Contrary to the above two cases, the ^{22}acdAla mutant showed no change of fluorescence intensity on the addition of biotin. We believe that the ^{22}acdAla mutant still keeps biotin binding activity, because the dot blotting shows similar biotin binding activity as the ^{83}acdAla mutant as shown in Figure 1c. As will be indicated later in Figure 7, the ^{22}acdAla group is not much more separated from the bound biotin than the acdAla groups in the other two mutants. Since all three mutants bind biotin and the distances between acdAla unit and the bound biotin are not much different, the insensitivity of the ^{22}acdAla mutant cannot be explained simply. Presumably, the ^{22}acdAla mutant does not undergo biotin-induced conformational changes that cause large enough change of the polarity around the fluorophore. In any case, it must be emphasized that the proper selection of the fluorophore positions is very important for sensitive detection of small molecules by labelled proteins.

Similar to the ^{22}acdAla case, thermally denatured ^{83}acdAla mutant did not respond to biotin. In this case, however, the insensitiveness must be interpreted in terms of the loss of biotin binding activity.

It may be mentioned again that when an acdAla was incorporated into proper positions, the mutant streptavidin can detect pM concentrations of biotin from fluorescence measurement.

Figure 5 shows the quantum yields of Boc-acdAla-OH in various solvents of different dielectric constants. The fluorescence intensity decreased with the decrease of the solvent polarity. This result suggests that the decrease of polarity around the acridonyl group by the bound biotin is a major reason for the decrease of fluorescence intensity.

2.4. Fluorescence decay analysis of various mutants of acdAla-streptavidin in the absence and presence of biotin

Fluorescence decay curves of ^{83}acdAla streptavidin in the presence of different concentrations of biotin are shown in Figure 6. The decay curves were fitted to single or double-exponential functions as collected in the

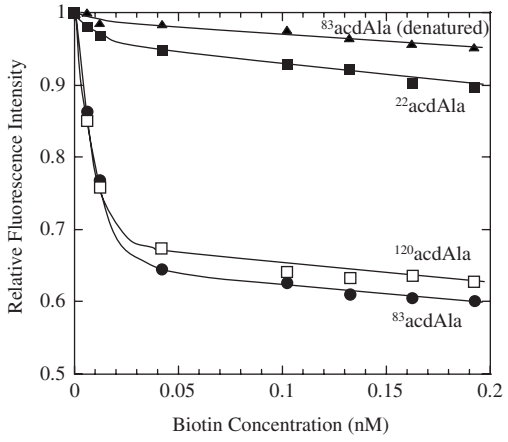


Figure 4. Fluorescence titration curves of various acdAla-streptavidins by biotin in 50 mM $NaHPO_4$, 300 mM NaCl solution containing 0.02% polyethyleneglycol at pH = 7.0, 25 °C. Excitation wavelength was 405 nm.

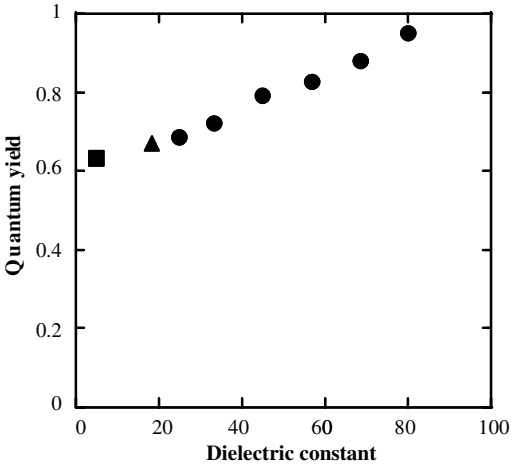


Figure 5. Fluorescence quantum yields of Boc-acdAla-OH in various solvents of different dielectric constants: (solid circles) ethanol/water, 100/0, 80/20, 60/40, 40/60, 20/80, 0/100; (solid triangle) *n*-butanol; (solid square) dioxane.

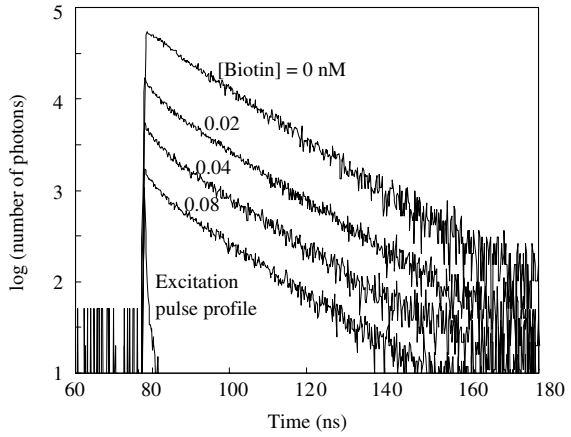


Figure 6. Fluorescence decay curves of ^{83}acdAla-streptavidin in the absence and presence of biotin (0.02 nM, 0.04 nM, and 0.08 nM).

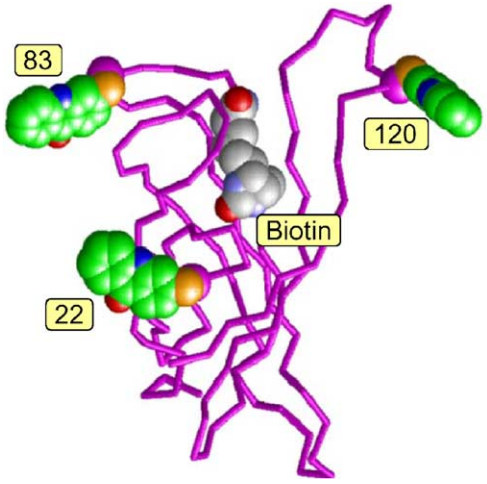


Figure 7. Computer-predicted orientations of the acdAla units at three different positions in streptavidin. The three acdAla units are shown collectively in this figure. The main chain conformation was not altered by the introduction of the acdAla unit.

Supplementary data. In the absence of biotin, the decay curve fitted to a single exponential function indicating that all fluorophores are situated under the same environment. The decay time was 15.1 ns, that is close to the decay time of Boc-acdAla-OH (16.0 ns). In the presence of biotin, a short-lived component (decay time $\approx$ 3 ns) appeared in the decay curve, indicating some portion of the acridonyl group is quenched by the bound biotin. Least-squares fitting of the double exponential decay curve indicated that the fraction of the short-lived component was 10 ± 1%. The latter fraction is substantially smaller than the percentage of quenching in the fluorescence titration curve (35%) shown in Figure 4. The smaller quenching fraction in the decay curve may be interpreted in terms of an additional quenching process that does not fluoresce at all.

2.5. Prediction of the orientations of the acridonyl groups in mutant streptavidins

To discuss about the quenching process by the bound biotin, information on the orientations of acridonyl fluorophores in the mutant proteins is very important. The orientations were predicted by a software (PRO-CON) for molecular mechanics calculations on mutant proteins that contain nonnatural amino acids.[15,16] Starting from the X-ray crystallographic structure of biotin-bound tetrameric streptavidin,[17] an acdAla unit was introduced at each specific position. Minimum-energy conformations were searched by varying only rotational angles for side groups of amino acids that are within 8 Å from one of the atoms of acdAla unit. Main chain conformation was kept unchanged during the energy-minimization process. This may be partly justified by the fact that all three mutants retained the biotin-binding activity. The computer-predicted conformation is shown in Figure 7.

2.6. Fluorescence sensing of lysozyme with mutant camel single-chain antibody incorporated with a single acdAla unit at various positions

Fluorescent mutants of cAb's against hen lysozyme were also synthesized. In this case an acdAla unit was introduced at the 103rd, 106th, 107th, and 123rd positions. Figure 8 shows fluorescence titration curves against lysozyme concentration.

In the cases of the ^{106}acdAla and ^{107}acdAla mutants, the fluorescence was sensitively quenched with the addition of lysozyme, indicating that strong antigen binding activity is retained for the two mutants. The extent of the quenching was, however, smaller than the case of streptavidin/biotin pair. As has been observed in the cases of streptavidin mutants, the binding and/or quenching depends substantially on the positions of acdAla unit. The ^{103}acdAla mutant showed a less sharp titration curve, suggesting that the antigen binding is somewhat suppressed by the introduction of acdAla unit.

The ^{123}acdAla mutant did not show clear quenching on the addition of the antigen. This may suggest either the loss of the antibody activity or the insensitiveness of the acridonyl group at the 123rd position against the bound biotin. Since the titration curves show small change of

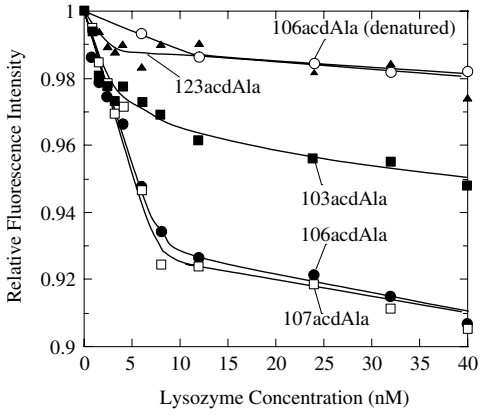


Figure 8. Fluorescence titration curves of various acdAla-cAbs in 50 mM $NaHPO_4$, 300 mM NaCl solution containing 0.02% polyethyleneglycol at pH = 7.0, 25 °C, in the presence of various amounts of hen lysozyme. Excitation wavelength was 385 nm.

the curvature, the latter interpretation may be favored at this moment. The top curve of Figure 8 is the titration curve for a thermally denatured ^{106}acdAla-cAb. As in the case of thermally denatured mutant of streptavidin, the insensitiveness may be explained in terms of the loss of antigen binding activity.

2.7. Prediction of the orientations of the acridonyl groups in mutant camel single-chain antibodies

Orientations of the acridonyl groups in mutant cAbs were also predicted, starting from the X-ray crystallographic structure of the antibody–antigen complex.[18] The computer-predicted orientations are shown in Figure 9.

The positions and orientations of the acridonyl groups in Figure 9 are consistent to the titration curves in Figure 8. Both the ^{106}acdAla and ^{107}acdAla mutants responded sensitively to the antigen. In these two mutants the fluorophore is located close to the antigen binding site. A less sensitive titration curve of ^{103}acdAla mutant may be interpreted in terms of a reduced antigen binding activity by the fluorophore in the antigen binding site. Insensitiveness of the ^{123}acdAla mutant is clearly due to the far separation of the fluorophore from the bound antigen.

3. Conclusions

2-Acridonylalanine can be efficiently incorporated into specific positions of a protein by the chemical aminoacylation and the four-base codon/anticodon strategy. The acridonyl fluorophore is photoexcited by blue lasers and durable to prolonged photoirradiation. It is sensitive to small changes in polarity. Its fluorescence decay time is on the range easy to measure.

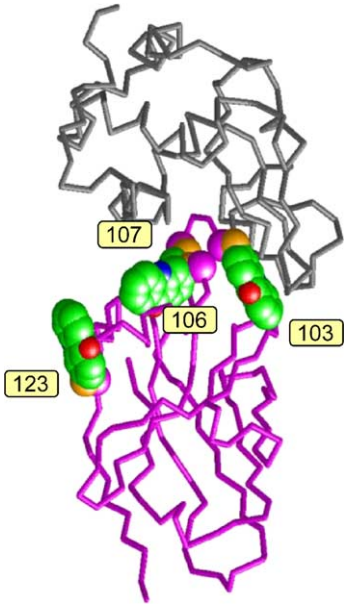


Figure 9. Computer-predicted orientations of the acdAla units at four different positions in camel single-chain antibody. The four acdAla units are shown collectively in this figure. The main chain conformation is not altered by the introduction of the acdAla unit.

Both the streptavidin and cAb mutants that contain acdAla units demonstrated that the position of fluorophore is crucially important for sensitive detection of small molecules and antigens. Position-specific incorporation of nonnatural amino acid is currently the only technique to locate a fluorophore to the sensitive positions. Acridonylalanine is currently the best amino acid for position specific fluorescence labelling with blue-laser excitation.

4. Experimental

4.1. Synthesis of pdCpA-acdAla and its ligation to tRNA (-CA)

Synthesis of Boc-acdAla has been reported.[14] The amino acid (32 mg, 0.084 mmol) was cyanomethylated by adding chloroacetonitrile (50 μL, 0.8 mmol) and triethylamine (150 μL, 1.1 mmol) in 300 μL of acetonitrile. After stirring for 24 h at room temperature, the reaction mixture was diluted with ethyl acetate and washed with 4% $NaHCO_3$, 5% $KHSO_4$, and then with saturated NaCl. The organic phase was dried over $MgSO_4$ and the solvent was evaporated. The resulting product was dried under vacuum to yield 18 mg (50%) of yellow powder. Aminoacylation of pdCpA was carried out as previously described.[13] Boc-acdAla cyanomethyl ester (1.1 μmol) was added to pdCpA tetrabutylammonium salt (0.22 μmol) in 5 μL of DMF and the resulting solution was incubated at 37 °C for 18 h. The reaction was monitored by reverse-phase HPLC [Waters μBondasphere C18, flow rate 0.6 mL/min, linear gradient from 100% 0.1 M $AcONH_4$ (pH 4.5) to 100% MeOH over 50 min]. The Boc-protected aminoacyl-pdCpA was collected as a precipitate by adding ether, washed with ether, and then dried under vacuum. To remove Boc group, the product was dissolved in 100 μL of trifluoroacetic acid (TFA) and the resulting solution was incubated for 10 min on ice. TFA was flushed off with N_2 gas and the resulting pellet was washed with ether. The product, pdCpA-acdAla, was identified by mass spectroscopy (Mariner ESI-TOF mass spectrometer): calcd for $C_{35}H_{37}O_{15}N_{10}P_2$ ($M-H^-$) 899.6843; found 899.6801.

The pdCpA-acdAla was ligated with a tRNA that lacks the CA dinucleotide unit at the 3′-terminal by T4 RNA ligase as previously reported.[13] The tRNA sequence was taken from yeast phenylalanine tRNA with an insertion of a CCCG anticodon at the anticodon loop.[13]

4.2. In vitro synthesis of nonnatural mutants of streptavidin and camel single-chain antibody

The chemically aminoacylated tRNA in aqueous solution was washed with acidic phenol and with chloroform. Then, it was precipitated with ethanol. The purified tRNA-acdAla was put into an *E. coli* S30 in vitro protein synthesizing system that contained mRNA encoding mutant streptavidin or mutant cAb with a CGGG 4-base codon at various positions. The whole base sequence of streptavidin has been reported

previously[19] and that of the single chain camel antibody is listed in Supplementary data. The sequences of the two proteins contained a T7 tag at the N-terminal and a His_6 tag at the C-terminal. The in vitro translation mixture (10 μL) contained 55 mM Hepes-KOH, pH 7.5, 210 mM potassium glutamate, 6.9 mM ammonium acetate, 12 mM magnesium acetate, 1.7 mM DTT, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% polyethyleneglycol-8000, 35 μg/mL folinic acid, 0.1 mM each amino acid, 16 μg (21 pM) mRNA, and 2 μL of *E. coli* S30 extract. Concentration of $tRNA_{CCCG}$-acdAla was about 50 μM. The product was analyzed on Western blotting using anti-T7 tag antibody. Protein yields were quantified by comparing the band intensity of the mutant obtained in the presence of the acdAla-tRNA to those of serially diluted wild-type streptavidin. Biotin binding activities of mutant streptavidins were evaluated by a dot blot analysis using biotinylated alkaliphosphatase as described previously.[13]

For fluorescence and fluorescence decay measurements, the protein products of the in vitro synthesis were applied to TALON metal affinity column (15 μL) equilibrated with a phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0) for mutant streptavidins or with a Tris–HCl buffer (20 mM Tris–HCl, 100 mM NaCl, pH 7.0) for mutant cAbs. The column was washed with 40×200 μL of phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, 5 mM purified imidazole, pH 7.0) for streptavidins or with 20×200 μL of Tris–HCl buffer (20 mM Tris–HCl, 100 mM NaCl, pH 8.0). Full-length streptavidins were eluted with a 30 μL of phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, 500 mM purified imidazole, and 0.1% PEG8000, pH 7.0). Full-length cAbs were eluted with a 30 mL of Tris–HCl buffer (50 mM Tris–HCl, 300 mM NaCl, 225 mM imidazole, and 0.1% PEG4000, pH 7.0). The eluents were diluted with the same buffer without imidazole 10 times for fluorescence measurements.

4.3. Measurements

Fluorescence spectra were recorded on a Spex Jobin-Yvon Fluoromax2 instrument. Excitation wavelength was 385 or 405 nm. Slit widths for excitation and for emission were 3 nm. For photodurability studies, fluorophore solutions of 0.05 absorbance at their absorption maxima in a 1×1 cm^2 cuvette were continuously irradiated on a Jasco FP777 instrument (excitation slit width = 3 nm) and the fluorescence intensity was measured occasionally during 1 h irradiation. No correction for different light intensities at different wavelengths has been made.

Fluorescence decay curves were measured on a time-correlated single photon counting instrument equipped with a blue-laser (415 nm) working under pulsed mode (pulse width = 70 ps, PicoQuant, LDH400). Photons of fluorescence were detected by a microchannel photomultiplier tube (Hamamatsu) working at −30 °C. The decay curves were fitted to single-exponential or double-exponential functions by a home-made iterative reconvolution program.[20]

Acknowledgements

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Supplementary data

Supplementary data is available for a complete base sequence of cAb, amino acid sequence of cAb, and results of least-squares analysis of the decay data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.03.014.

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