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Position-Specific Incorporation of a Fluorophore−Quencher Pair into a Single Streptavidin through Orthogonal Four-Base Codon/Anticodon Pairs

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Abstract: Four-base codon strategy was applied to incorporate a fluorophore−quencher pair into specific positions on a single protein; β-anthraniloyl-L-α,β-diaminopropionic acid (atnDap) was employed as a fluorophore and *p*-nitrophenylalanine (ntrPhe) as a quencher. Their positions were directed by the CGGG/CCCG and GGGC/CCCG four-base codon/anticodon pairs and two doubly mutated streptavidins, i.e., (^{52}atnDap, ^{84}ntrPhe) and (^{54}ntrPhe, ^{84}atnDap) mutants were synthesized through *Escherichia coli* in vitro protein synthesizing systems. Intramolecular photoinduced electron transfer (ET) was observed as the decrease of intensity in steady-state fluorescence spectroscopy and as the shortening of fluorescence decaytimes. The quenching data indicated that the ET rate reflects the detailed structure of the protein.

Position-specific incorporation of nonnatural amino acids into proteins allows us to introduce specialty functions into three-dimensional polypeptide frameworks.[1,2] We have found several four-base codon/anticodon pairs that are orthogonal to each other and to the existing three-base codon/anticodon pairs.[3−5] The orthogonal four-base codons can assign different nonnatural amino acids to individual positions along a polypeptide chain, leading to build a functional motif on a protein.

One of our goals by using this technique is to build a pathway for electron transfer (ET) on proteins. Since the ET and other photoprocesses are very sensitive to the distances between donors and acceptors of the order of several angstroms, the position-specific incorporation of multiple amino acids will be a powerful approach to design and build artificial ET pathways. For this approach to be successful, however, one must have detailed information on the proper chromophore arrangements for effective ET in proteins. In our previous study on the distance dependence of ET in a protein, a single L-*p*-nitrophenylalanine (ntrPhe) was introduced as the electron acceptor or quencher at various positions of streptavidin and site-to-site photoinduced electron transfer was observed from biotin-linked pyrenylalanine to the quencher.[6] Since the pyrenyl group has been introduced into streptavidin as the biotin derivative, there remained some ambiguity on the location and orientation of the pyrenyl group. A more reliable distance dependence has been studied on model peptides with pyrenyl−nitrophenyl pairs that are covalently linked to α-helical[7] and β-sheet scaffolds.[8] The latter peptides have been synthesized by the solid-phase method that is, however, not applicable to larger polypeptide chains or proteins.

In this paper, we report the first attempt to introduce a fluorophore−quencher pair into specific positions on a single protein by incorporating two nonnatural amino acids. The positions of the amino acids were directed by using a set of orthogonal four-base codon/anticodon pairs. So far, two orthogonal four-base codon/anticodon pairs, (CGGG/CCCG, AGGU/ACCU)[4] and (CGGG/CCCG, GGGU/ACCC),[5] have been used successfully to incorporate two different amino acids with higher efficiency in the latter set. In this study we examined a third set, (CGGG/CCCG, GGGC/GCCC), for incorporating a β-anthraniloyl-L-α,β-diaminopropionic acid (atnDap)[9] as a fluorescent amino acid and a *p*-nitrophenylalanine (ntrPhe) as a quencher amino acid into single streptavidin. Two doubly mutated streptavidins, i.e., (^{52}atnDap, ^{84}ntrPhe) and (^{54}ntrPhe, ^{84}atnDap), were synthesized. Streptavidins that contain only an atnDap unit at the 52nd and 84th positions, respectively, were

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also prepared as references. We measured steady-state fluorescence spectra as well as fluorescence decay curves of the doubly mutated streptavidin and compared the results with those of the reference proteins.

Experimental Section

Aminoacyl tRNAs. Boc protected atnDap was synthesized in our laboratory.[9] It was linked to the 3′-OH group of a mixed dinucleotide, pdCpA, and then the Boc group was removed by treatment with trifluoroacetic acid. The aminoacylated pdCpA was ligated with a tRNA that lacks a 3′-terminal pCpA unit and contains a CCCG or GCCC four-base anticodon, by T4 RNA ligase.[6] The resulting atnDap-$tRNA_{CCCG}$ and atnDap-$tRNA_{GCCC}$ were used to incorporate the fluorophore at the position of the CGGG and GGGC four-base codons, respectively. Similarly, ntrPhe-$tRNA_{CCCG}$ and ntrPhe-$tRNA_{GCCC}$ were synthesized and used to incorporate the quencher.[6]

Preparation of mRNA That Encodes a Doubly-Mutated Streptavidin Carrying a T7 Tag at the N-Terminal and a His_6 Tag at the C-Terminal. A synthetic gene for streptavidin was purchased from R&D Systems Europe, and a PCR mutagenesis was performed to replace two specific codons of the streptavidin gene by the four-base codon CGGG and GCCC.[6] A plasmid (pGSH) was designed to contain a T7 promoter, a T7 tag, a streptavidin including double four-base codons, a His_6 tag, and a T7 terminator.[6] The complete sequence of the gene is shown in the Supporting Information. The corresponding mRNAs were synthesized in vitro by T7 RNA polymerase.[6]

Expression and Detection of Each Doubly Mutated Streptavidin in Vitro. The protein biosynthesis was carried out as follows. AtnDap-$tRNA_{CCCG}$ and ntrPhe-$tRNA_{GCCC}$ were added into an in vitro biosynthesizing system of *E. coli* S30 lysate (Promega) together with a mRNA that contains ^{52}CGGG, ^{84}GGGC four-base codons to synthesize the (^{52}atnDap, ^{84}ntrPhe) mutant.[4,6] Similarly, the (^{54}ntrPhe, ^{84}atnDap) mutant was synthesized by adding atnDap-$tRNA_{GCCC}$ and ntrPhe-$tRNA_{CCCG}$ together with a mRNA that contains 54CGGG, ^{84}GGGC four-base codons. The synthesis of the full-length mutant streptavidins was confirmed by SDS-PAGE followed by Western blot analysis using an anti-T7 tag antibody (Novagen) and alkaliphosphatase-labeled anti-mouse IgG (Promega).

Biotin Binding Ability of Doubly Mutated Streptavidins. 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 alkaliphosphatase (Zymed) as described previously.[6] The immunoassay was repeated at least three times. The biotin-binding activity was also measured by the change of fluorescence polarization of an FITC-labeled biotin. The fluorescence polarization was measured on a BEACON 2000 system (PanVera Corp., Madison, WI) equipped with optical filters of 490 nm for excitation and 520 nm for emission. Portions of the in vitro reaction mixture were sequentially added to 100 μL of 1 nM biotin−FITC solution in TBS buffer containing 0.1% PEG8000 at pH 7.0. The solution was incubated for 5 min at 25 °C before each measurement.

Affinity Purification of Doubly Mutated Streptavidins. For fluorescence studies, a large-scale production of mutant streptavidin was carried out using 20 μL of S30 lysates for each batch (100 μL). The reaction mixture was centrifuged at 15 000*g* for 10 min; then the supernatant was loaded onto 10 μL of TALON column (Clontech) equilibrated with buffer A containing 50 mM sodium phosphate and 300 mM NaCl at pH 7.0. Only the full-length mutant proteins which contain both atnDap and ntrPhe carry the C-terminal His_6 tag and, therefore, bind to the TALON column. The column was washed with seven 1 mL portions of buffer B containing 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 concentration of the purified mutant was determined by the densitometry of the Western blotting. Solutions of wild-type streptavidin of known concentrations ($\epsilon_{282} = 4.3 \times 10^4$ cm^{-1} L mol^{-1}) were used as the standard.

Fluorescence Spectra and Fluorescnce Decay Measurements of Mutant Streptavidins. Fluorescence spectra were recorded on a Spex-Joan-Yvon Fluoromax2 spectrophotometer. Slit widths were 1.5 nm for excitation and 5 nm for emission, respectively. The mutant solution of known concentration was diluted by nine portions of buffer A and transferred into a microcuvette under argon. Spectra were measured with excitation at 343 nm. The preparation of sample solution and the measurement were repeated independently at least three times. Experimental errors represent the standard deviations from these independent experiments.

Fluorescence decay curves were measured on a time-correlated single-photon counting apparatus equipped with a frequency-doubled mode-locked laser system (Spectra Physics, Millenia Xs-Tsunami system). The excitation wavelength was 347 nm. The decay curves were analyzed by an iterative reconvolution program.

Prediction of the Orientations of the Side Groups of atnDap and ntrPhe Units in the Mutant Streptavidins. The orientations of the atnDap and ntrPhe groups in the mutants were predicted from molecular mechanics calculations on a software (PROCON) as described previously.[6,10] From the coordinates of the predicted minimum-energy conformation, the closest aromatic carbon−aromatic carbon distance was obtained and used as the edge-to-edge distance r_{ee} between the atnDap and ntrPhe groups.

Results and Discussion

Expression of Doubly Mutated Streptavidins. AtnDap was chosen as a fluorescent amino acid and ntrPhe was chosen as a quencher amino acid. As we reported previously, both non-natural amino acids have been incorporated into streptavidin in high efficiencies with minimum conformational constraint.[9,11] The positions of the atnDap−ntrPhe pair were chosen to be 52nd−84th and 84th−54th, respectively, because of their insensitiveness to the biotin−binding activity. The former mutant was synthesized by adding both the atnDap-$tRNA_{CCCG}$ and ntrPhe-$tRNA_{GCCC}$ to an *E. coli* in vitro biosynthesizing system together with a mRNA that contains four-base codons at two positions, ^{52}CGGG and ^{84}GGGC. The second mutant was synthesized by adding both the atnDap-$tRNA_{GCCC}$ and ntrPhe-$tRNA_{CCCG}$ together with a mRNA that contains ^{54}CGGG, ^{84}GGGC four-base codons. The complete sequence of the DNA is shown in the Supporting Information. Synthesis of the mutant streptavidins was confirmed by SDS-PAGE followed by Western blot analysis using an anti-T7 tag antibody and alkaliphosphatase-labeled anti-mouse IgG.

As shown in Figure 1a, full-length streptavidins were synthesized only in the presence of both the atnDap-$tRNA_{CCCG}$ and ntrPhe-$tRNA_{GCCC}$ (lane 2) or both the atnDap-$tRNA_{GCCC}$ and ntrPhe-$tRNA_{CCCG}$ (lane 5). The expression of full-length streptavidin indicates that both the four-base codons on the mRNA were successfully translated by the corresponding four-base anticodons, resulting in the incorporation of the two nonnatural amino acids into specified positions.[2,3] No full-length protein was detected in the absence of aa-$tRNA_{CCCG}$ (lanes 4 and 6), indicating that the four-base codon CGGG was decoded only by aa-$tRNA_{CCCG}$. In the absence of aa-$tRNA_{GCCC}$, the ^{84}GGGC sequences were not decoded as the four-base codon (lane 3), except for a small spot at the full-length protein observed in lane 7.

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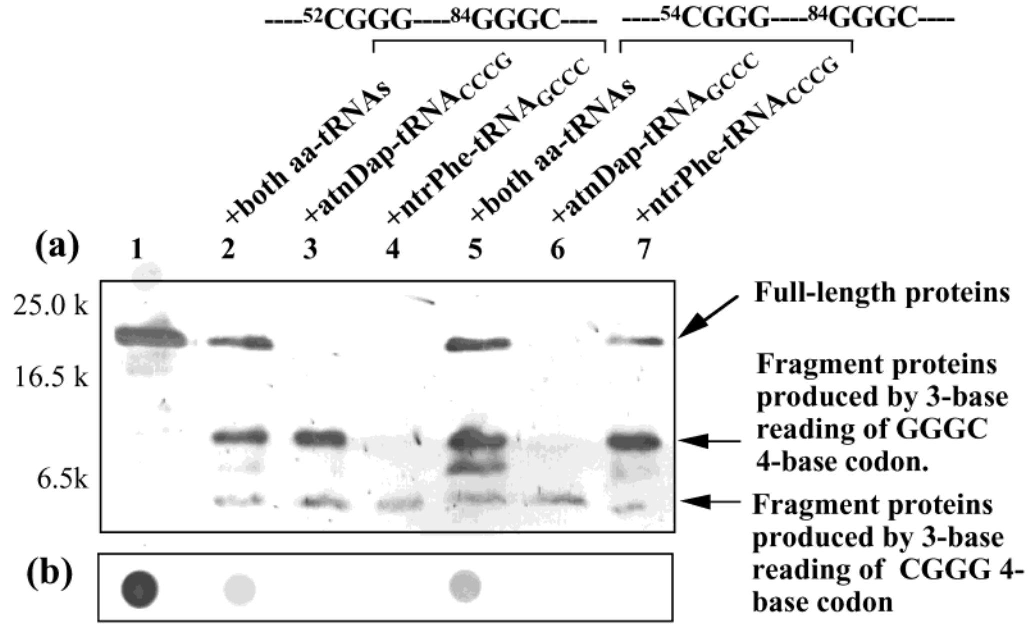

Figure 1. (a) Western and (b) dot blot analysis of the translation products of various mRNAs. Wild-type streptavidin mRNA (lane 1 and spot 1). Mutated mRNA containing the CGGG and GGGC four-base codons at the 52nd and 84th positions, respectively, added with atnDap-tRNA$_{CCCG}$ (lane 3 and spot 3), with ntrPhe-tRNA$_{GCCC}$ (lane 4 and spot 4), and with both aminoacyl tRNAs (lane 2 and spot 2). Mutated mRNA containing the CGGG and GGGC four-base codons at the 54th and 84th positions, respectively, added with atnDap-tRNA$_{GCCC}$ (lane 6 and spot 6), with ntrPhe-tRNA$_{CCCG}$ (lane 7 and spot 7), and with both aminoacyl tRNAs (lane 5 and spot 5).

In these cases, a large amount of the fragment proteins were formed. According to the full sequence, if the ^{52}CGGG or the ^{54}CGGG was decoded as a three-base codon, the reading frame will be shifted forward by one base and the protein synthesis will terminate at the ^{60}TA$\underline{\text{T}}$-$^{61}\underline{\text{GA}}$C stop codon. Similarly, if the ^{84}GGGC sequence was decoded as a three-base codon, the protein synthesis will terminate at the ^{100}GC$\underline{\text{T}}$-$^{101}\underline{\text{GA}}$G position.

A small spot of full-length protein was detected in the absence of atnDap-tRNA$_{GCCC}$ (lane 7), although efficiency of the undesired translation is not high and a much larger amount of truncated protein is observed. As described below, the undesired full-length protein did not show biotin-binding activity on the dot blot analysis (Figure 1b, spot 7). Therefore, we tentatively assign it as a product of three-base decoding of ^{84}GGGC by endogenous Gly-tRNA$_{CCC}$ followed by a four-base decoding of ^{98}GG$\underline{\text{C}}$ $^{99}\underline{\text{GGT}}$ sequence by a large amount of ntrPhe-tRNA$_{CCCG}$ added into the mixture, before the encounter of the ^{100}GC$\underline{\text{T}}$-$^{101}\underline{\text{GA}}$G stop codon.

Biotin Binding Ability of Doubly Mutated Streptavidins. The biotin-binding activities of the full-length mutants were examined by dot blot analysis for the reaction mixture of the in vitro synthesis using biotin-linked alkaliphosphatase. As shown in Figure 1b, both doubly mutated streptavidins retained biotin-binding activity (spots 2 and 5).

The biotin binding activity was further confirmed by the change of fluorescence polarization of FITC−biotin conjugate with the addition of the doubly mutated streptavidins. The double mutants with a histidine tag at the C-terminal were purified using TALON metal affinity resin. After loading and washing, the mutant proteins were eluted with a 500 mM imidazole solution. As shown in Figure 2, the fluorescence polarization of FITC−biotin gradually increased with the addition of either doubly mutated streptavidin. The binding constants evaluated from the comparison of the titration curves with that of the wild-type streptavidin ($K \sim 10^{15}$) were on the order of 10^8 M^{-1} for both double mutants. The observation of the biotin binding at nM concentration range and the high enough binding constants suggest that the three-dimensional structure is essentially retained, despite the incorporation of two nonnatural amino acids.

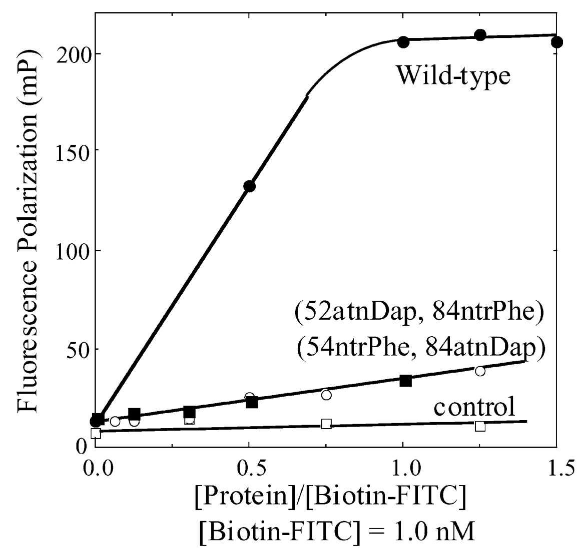

Figure 2. Fluorescence polarization of biotin−FITC in the presence of various amounts of proteins. [biotin−FITC] = 1.0 nM, λ_{ex} = 490 nm in 50 mM phosphate buffer, 300 mM NaCl, pH = 7.0 at 25 °C.

Naturally, incorporation of two nonnatural amino acids increases the possibility of unfolding.[12] Indeed, other double mutants such as (^{52}atnDap, ^{83}ntrPhe) were synthesized as full-length proteins but did not show biotin binding activity. The biotin binding activity observed for the (^{54}ntrPhe, ^{84}atnDap) and (^{52}atnDap, ^{84}ntrPhe) mutants is therefore noteworthy. A more

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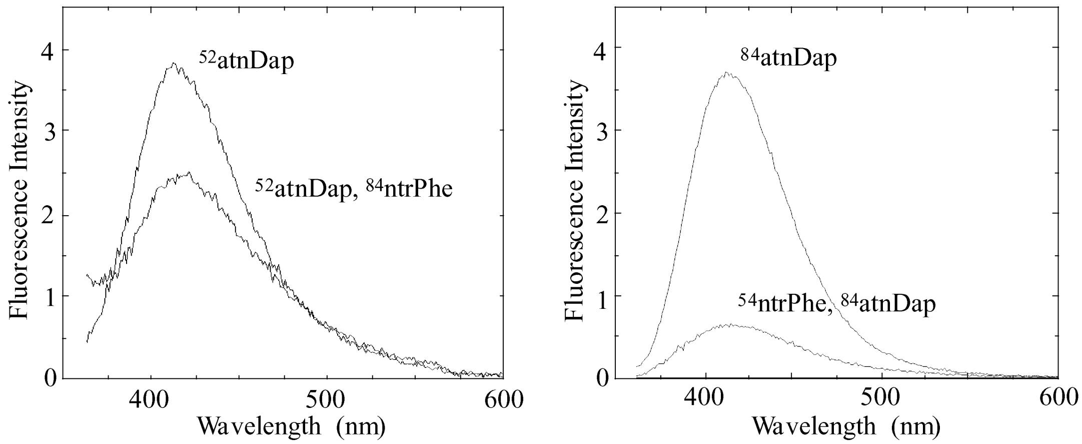

Figure 3. (a) Fluorescence spectra of the ^{52}atnDap single mutant (upper) and the (^{52}atnDap, ^{84}ntrPhe) double mutant (lower). (b) Fluorescence spectra of the ^{84}atnDap single mutant (upper) and the (^{54}ntrPhe, ^{84}atnDap) double mutant. λ_{ex} = 343 nm in 50 mM phosphate buffer, 300 mM NaCl, pH = 7.0 at 25 °C.

Table 1. Edge-to-Edge Distances between atnDap and ntrPhe Units (r_{ee}, Å), Fluorescence Intensities (ϕ), Decaytimes (τ_i, ns) with Their Weights (w_i), and χ^2 Values for the Curve Fitting

mutant	r_{ee} (Å)	ϕ^a	τ_1	w_1	τ_2	w_2	$\langle\tau\rangle$	χ^2
^{52}atnDap		1.0	1.1	0.22	8.0	0.78	6.4	1.2
(^{52}atnDap, ^{84}ntrPhe)	12.8	0.27	1.0	0.39	5.6	0.61	3.8	1.2
^{84}atnDap		1.0	1.1	0.17	7.9	0.83	6.7	1.0
(^{54}ntrPhe, ^{84}atnDap)	8.9	0.54	1.2	0.41	6.4	0.59	4.2	1.1
Boc-atnDap			7.6				7.6	1.2

^{a} Fluorescence intensity at 412 nm (λ_{ex} = 343 nm) divided by the intensity of the single mutant.

systematic search for unsusceptible positions to amino acid replacements will need construction of a library of mutant proteins that contain nonnatural amino acids at random positions. The random replacement with a nonnatural amino acid will become possible by a replacement of three consecutive bases at random positions on a target DNA, followed by insertion of a specific four-base codon. The random insertion/deletion (RID) mutagenesis has been reported recently.[13]

Fluorescence Behavior of Doubly Mutated Streptavidins Carrying an atnDap−ntrPhe Fluorophore−Quencher Pair. Steady-state fluorescence spectra were measured for the purified proteins. Figure 3 shows fluorescence spectra of the atnDap unit in the single and double mutants. The observed fluorescence intensities have been normalized by protein concentrations evaluated from densitometric analysis of Western blotting. The normalized fluorescence intensities (ϕ) are collected in Table 1. Fluorescence intensity decreased with the introduction of the ntrPhe unit in both double mutants. As shown in the Supporting Information, the nitrophenyl group shows little absorption above 350 nm and little spectral overlap with the fluorescence spectrum of β-anthraniloyl group. The spectral property ensures that the fluorescence quenching is not due to an energy transfer but to an ET from the excited anthraniloyl group to a nitrophenyl group. The ET quenching must be an intramolecular process because no intermolecular quenching was observed under nM order concentrations of a low-molecular-weight quencher.

Fluorescence Decay Curves of Doubly Mutated Streptavidins. Fluorescence decay analysis provides more reliable information on the ET quenching process, because the decay data do not depend directly on protein concentrations. The decay curves are shown in Figure 4. The decay curves of double mutants as well as those of single mutants fitted to two-component exponential curves. The decay times are summarized in Table 1. In both double mutants, introduction of a ntrPhe unit accelerated the fluorescence decay of atnDap unit and the longer decay time is more susceptible to the introduction of quencher. Since the longer decay times of the single mutants are close to the decay time of a low-molecular-weight model compound (Boc-atnDap-OH), they may be assigned to the fluorophores that are exposed to solvent. Because the longer decay time is the major component, the atnDap unit in the mutants must be exposed to solvent. The shorter and minor component may then be assigned to the fluorophores located in some abnormal environment, for example, in unfolded proteins.

From the shortening of longer decay times, the ET rate constants were calculated as $k_{ET} = \tau^{-1} - \tau_0{}^{-1}$, where τ_0 is the longer decay time of the single mutant and τ is that of the double mutant. The ET rate constants are $k_{ET} = 3.0 \times 10^7$ s^{-1} for the (^{54}ntrPhe, ^{84}atnDap) mutant and 5.3×10^7 s^{-1} for the (^{52}atnDap, ^{84}ntrPhe) mutant, respectively.

Computer Prediction of the Chromophore Orientations on the Mutant Streptavidins. Molecular mechanics calculations were carried out for the side-chain orientations of the atnDap and ntrPhe units. The starting conformation was taken from an X-ray crystallographic structure of streptavidin tetramer[14] and only the side groups of the two nonnatural amino acids and those of the neighboring amino acids were rotated to minimize conformational energy. The main chain conformation was kept unchanged because the mutants retained biotin binding activity. The computer-predicted orientations of the nonnatural amino acids are illustrated in Figure 5. The atnDap units at the 52nd and 84th positions are exposed to solvent. This is consistent with the conclusion from the fluorescence decay data.

The fluorophore−quencher edge-to-edge distances were calculated from the predicted structure. Results are listed in Table 1. The ET rate constant of the (^{52}atnDap, ^{84}ntrPhe) mutant was higher than that of the (^{54}ntrPhe, ^{84}atnDap) mutant, whereas the edge-to-edge distance was longer in the former mutant.

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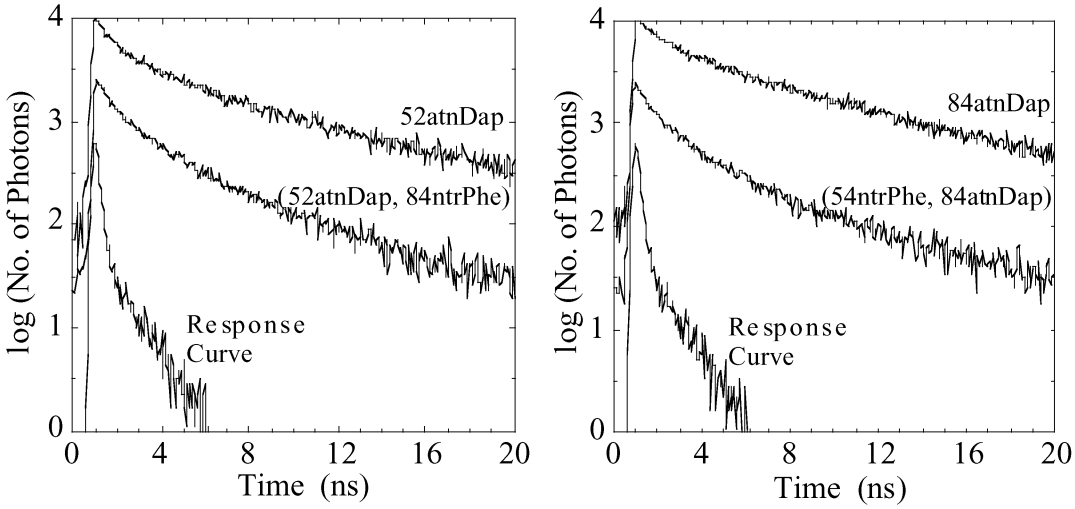

Figure 4. (a). Fluorescence decay curves of the ^{52}atnDap single mutant (upper) and the (^{52}atnDap, ^{84}ntrPhe) double mutant (lower). (b) Fluorescence decay curves of the ^{84}atnDap single mutant (upper) and the (^{54}ntrPhe,^{84}atnDap) double mutant (lower). λ_{ex} = 347 nm in 50 mM phosphate buffer, 300 mM NaCl, pH = 7.0 at 25 °C.

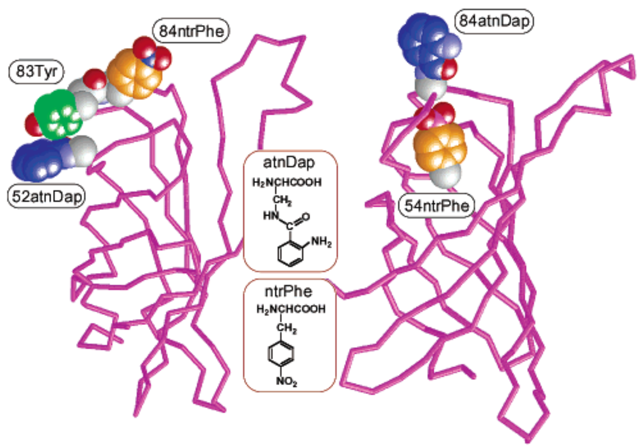

Figure 5. Computer-predicted conformation of the (^{52}atnDap, ^{84}ntrPhe) double mutant (left) and the (^{54}ntrPhe, ^{84}atnDap) double mutant (right).

A close look at detailed structures of the predicted conformation gives a plausible reason for this discrepancy. In the (^{52}atnDap, ^{84}ntrPhe) mutant (Figure 5, left), an aromatic group of the ^{83}Tyr unit is lying on the ET path that may assist the ET process. On the (^{54}ntrPhe, ^{84}atnDap) mutant (Figure 5, right), however, an electron-rich nitro group (NO_2) is lying on the ET path and this may alter the ET mechanism or retard the ET process. The detailed arrangements of the fluorophore, quencher, and other adjacent groups may regulate the ET process on a protein, although the discussion has to wait for X-ray structures of the mutants.

Conclusion

To conclude, a fluorophore−quencher pair was incorporated as nonnatural amino acids into streptavidin. The positions of the two nonnatural amino acids have been directed by an orthogonal combination of four-base codon/anticodon pairs, CGGG/CCCG and GGGC/CCCG. Intramolecular ET quenching was observed from the steady-state spectra as well as the decay measurements. The position-specific multiple incorporation of nonnatural amino acids was shown a powerful tool for building ET pathways on single proteins.

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Supporting Information Available: Complete sequences of the DNA for doubly mutated streptavidins and the spectral overlap of absorption of ntrPhe unit with the fluorescence of atnDap unit. This material is available free of charge via the Internet at http://pubs.acs.org.

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