

Donor–Donor Energy-Migration Measurements of Dimeric DsbC Labeled at Its N-Terminal Amines with Fluorescent Probes: A Study of Protein Unfolding**

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Fluorescence resonance energy transfer (FRET) is a powerful technique for the determination of distances between two fluorophores. The overall geometry of protein structures^[1–4] and the conformational changes of a molecule under different conditions can be studied by this method if appropriate sites of the molecule are labeled with fluorescence donor and acceptor probes. Nevertheless, it is rather difficult to specifically introduce two different fluorophore groups into one molecule,^[2] especially into a homodimeric biomacromolecule that has two identical reactive sites. Different from the conventional FRET technique, donor–donor energy migration (DDEM) takes advantage of certain fluorescence probes that display an overlap of their absorption and emission spectra and are therefore able to transfer energy between themselves.^[2–4] Energy transfer in this case is a reversible process and can be measured through analysis of the time-resolved depolarization of the fluorescence emission (as donor–donor energy migration results in additional depolarization). As only one type of probe is required, DDEM simplifies greatly not only the labeling operation but also the theoretical analysis and the time-resolved measurements and has been widely used to study the steady-state conformational changes of biomacromolecules.

DsbC (1), a member of the Dsb family in the periplasm of Gram-negative bacteria, is a thiol-protein oxidoreductase that displays molecular chaperone activity.^[5–7] The DsbC molecule is a V-shaped homodimer consisting of two 23.4-kDa subunits.^[8] Each subunit is composed of a C-terminal thiore-

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doxin-like domain and a N-terminal domain, which is responsible for dimerization and is essential for the chaperone activity of the molecule.^[8,9]

The V-shaped structure of homodimeric DsbC led us to apply the DDEM method to explore its unfolding and dissociation behavior and to understand further its structure–function relationship. In this context, the two N-terminal amino groups of DsbC are the sites of choice at which to link two identical probes; however, the labeling of other amino groups, such as ϵ -amino groups of lysine residues, and the nonspecific modification of groups other than amino groups could also occur. Several new methods for the introduction of fluorescent probes into proteins have recently been developed to improve the specificity of labeling.^[10–12] The most common approach is to engineer a pair of reactive cysteine residues to provide two thiol handles for conjugation.^[2,13] Alternatively, a ketone handle, produced through the introduction of an unnatural keto-containing amino acid, can be labeled with hydrazide-functionalized fluorophores with no observed cross-reactivity.^[1,13]

Herein, we describe a general method for the specific labeling of N-terminal groups through a transamination reaction, in which the N-terminal amino group of a protein molecule is converted into a reactive carbonyl group, which is then treated with a hydrazide-containing fluorophore. As the intermediate in transamination reactions involves the participation of an adjacent peptide bond, only the conversion of the terminal amino group occurs without modification of the internal amino groups on lysine residues.^[14–17] Subsequently, the conformational changes of dimeric DsbC during unfolding (induced by guanidine hydrochloride (GuHCl)) were studied by DDEM.

The fluorescent dye BODIPY FL (shown as the hydrazide derivative **2** in Figure 1) was employed as the probe owing to its high fluorescence quantum yield, its insensitivity to solvent polarity and pH, and its Förster radius of 57 Å.^[2,13,18] The N-terminal amino groups of **1** were modified as shown in Figure 1 by a) a transamination reaction in the presence of glyoxylate and CuSO₄,^[14,17] b) coupling of the product **3** with BODIPY FL hydrazide (**2**), and c) reduction of the imine groups to the more stable amine form **4** of the labeled product. Sodium cyanoborohydride instead of borane-pyridine was used as the reducing agent owing to its better selectivity for imines^[19] and the absence of quenching effects on the fluorescence from the BODIPY dye (data not shown).

In a similar procedure, **1** was also labeled by following the transamination step carried out in the absence of glyoxylate. As shown in Figure 2, the absorption spectrum of the protein modified in the *presence* of glyoxylate exhibits a main peak at 280 nm characteristic of native protein and a less intense band at 505 nm for the BODIPY moiety,^[15] whereas the absorption profile for the protein modified in the *absence* of glyoxylate shows only one band for native protein; this indicates that the BODIPY-labeled DsbC **4** can be prepared only through a transamination process carried out in the presence of glyoxylate.

To confirm further that the DsbC molecule had been specifically labeled with BODIPY, **4** was also examined by MALDI TOF mass spectrometry. A peak at m/z 47300, in

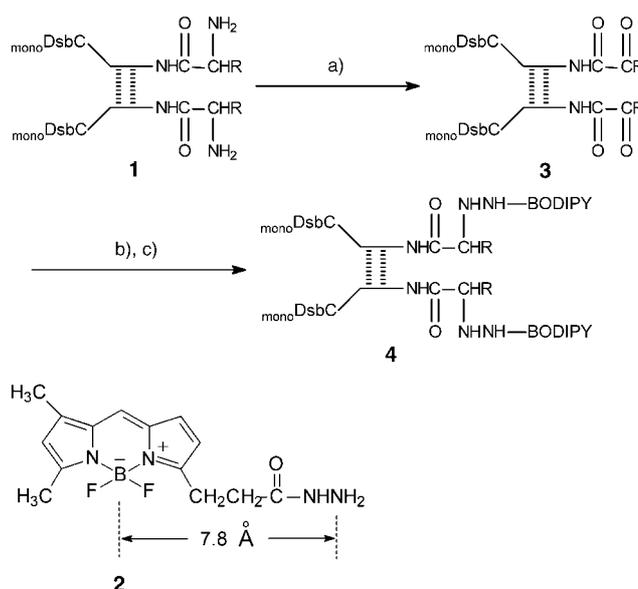


Figure 1. Specific modification of the N-terminal amino groups of dimeric DsbC (**1**): a) transamination reaction with glyoxylate; b) coupling with **2**; c) reduction with sodium cyanoborohydride. The separation between the central B atom of the BODIPY FL dye and the terminal N atom of the hydrazide group of the linker arm in **2** is 7.8 Å (calculated with CS Chem 3D software). BODIPY FL = 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid.

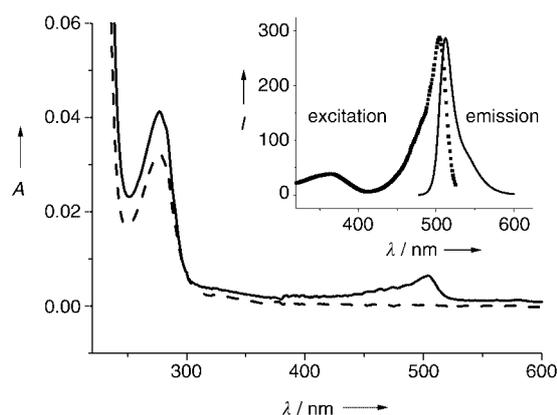


Figure 2. Absorption spectra of DsbC labeled with BODIPY. DsbC (**1**) was modified in the presence (solid line, **4**, 1.2 μM) and absence (dashed line, 1.0 μM) of glyoxylate. The inset shows the excitation ($\lambda_{\text{em}} = 535$ nm) and emission ($\lambda_{\text{ex}} = 467$ nm) spectra of **4** (4.1 μM); 5-nm excitation and emission slits were used.

agreement with the theoretical value of m/z 47410 expected for DsbC with two N-terminal BODIPY labels **4**, was detected with a mass error < 0.3%.^[20] Although the presence of a small amount of DsbC modified on only one N-terminal amino group cannot be ruled out, it should not affect the DDEM measurements, especially in dilute solution. The efficiency with which fluorophores are incorporated into DsbC is about 9%, which is ascribed to the limited accessibility of the N termini of the DsbC molecule.

The fluorescence spectra of **4** display an excitation band at 505 nm and an emission band at 510 nm (see Figure 2 inset),

which are almost the same as that for the free BODIPY dye^[13] and indicate that the attachment of the fluorophore to DsbC does not alter its spectral properties. On the other hand, **4** shows the same circular dichroism spectrum as that of the native DsbC,^[20] which suggests that the introduction of BODIPY does not affect the secondary structure of the protein. The native DsbC, the partially denatured DsbC formed in the presence of GuHCl (1.5 M), and the fully denatured DsbC formed in the presence of GuHCl (6 M) and dithiothreitol (0.1 M), all labeled with BODIPY, displayed near-identical fluorescence-decay profiles,^[20] which were fitted to a single exponential function with a satisfactory low value of χ^2 in the range of 1.17–1.38. The fluorescence lifetime (τ) in each of the three cases was about 6.7 ns (calculated based on $I(t) = Ae^{-(t/\tau)}$) and indicate that the fluorescence lifetime of BODIPY in BODIPY-labeled DsbC is unaffected by the extent of denaturing of the protein (Table 1). On the other hand, the decay rates of fluorescence anisotropy $r(t)$ show a variation with different extents of denaturing of DsbC (Figure 3). The initial decay of $r(t)$ of the fully denatured DsbC is much slower than that of the native DsbC. The fast decay of the fluorescence anisotropy from the native DsbC suggests that the observed emission is not from the originally excited BODIPY fluorophore. The other adjacent fluorophore in the same DsbC molecule could contribute to the observed emission by an energy-transfer mechanism and thereby lead to the fast depolarization. This is an experimental hallmark of donor–donor energy-migration processes.^[4]

The interfluorophore distance R is defined as the distance between the centers of two fluorophores and can be estimated

based on energy-transfer measurements.^[2–4,21] The rate ω of energy transfer between two interacting fluorophores is expressed by Equation (1) according to the Förster energy-transfer mechanism:^[2,3,21]

$$\omega = \frac{3}{2} \langle \kappa^2 \rangle \frac{1}{\tau} \left(\frac{R_0}{R} \right)^6 \quad (1)$$

(τ = fluorescence lifetime, R_0 = Förster radius ($57 \pm 1 \text{ \AA}$ for BODIPY),^[2,18] and $\langle \kappa^2 \rangle$ = orientation factor, for which an average value of $2/3$ is usually taken; the parameter ω obtained from DDEM measurements and the values of R estimated by Equation (1) are summarized in Table 1).

Table 1: Results from DDEM measurements^[a]

DsbC	ω [ns ⁻¹]	τ [ns]	R [Å]	R_c [Å]
Native	0.569	6.7	46	35
Partially denatured	0.141	6.7	58	47
Fully denatured	–	6.6	–	–

[a] The parameter ω was obtained from the best-fit curves based on $r(t) = A \exp(-2\omega t) + B$; the value of χ^2 for each fitting was in the range of 1.077–1.422. The interfluorophore distance R in **4** denatured to different extents was calculated according to Equation (1). R_c is the corrected value of R . The data quoted are the average of two independent experiments.

The calculated interfluorophore distances in the native and partially denatured DsbC are 46 and 58 Å, respectively, and contain a contribution from the length of the linker group of the BODIPY dye (Figure 1). Moreover, it is reasonable to

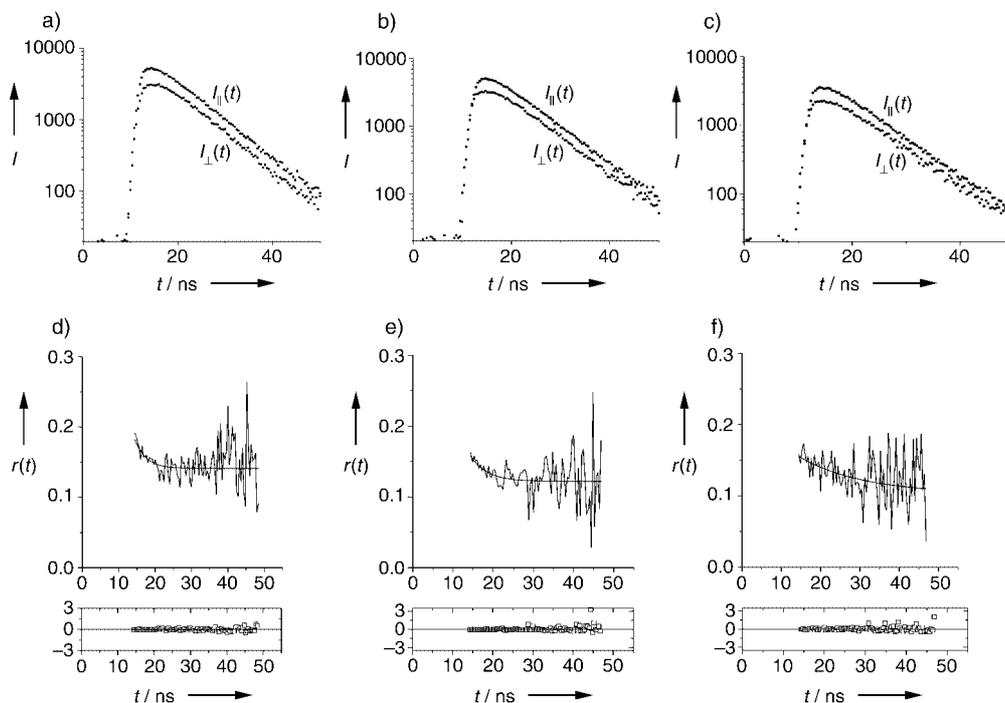


Figure 3. a)–c) Polarized fluorescence decays of $I_{\parallel}(t)$ and $I_{\perp}(t)$ and d)–f) anisotropy decays (along with the best-fit curves and the weighted residuals) of **4** (4.1 μM) at various extents of denaturing of the DsbC protein; a), d) native DsbC; b), e) denatured in GuHCl (1.5 M); c), f) denatured in GuHCl (6 M) with dithiothreitol (DTT, 0.1 M). $I_{\parallel}(t)$ and $I_{\perp}(t)$ represent the intensities of the fluorescence observed with the emission polarizer oriented parallel and perpendicular, respectively, to the excitation polarizer. All measurements were carried out at 273 K.

assume an averaged right-angled geometry between the two linker groups attached to the N termini of DsbC.^[21] The corrected value R_c for the native DsbC is thus 35 Å (Table 1), which is in agreement with the value of 29 Å measured from the crystal structure of the protein.^[8] Similarly, the corrected distance between the two N termini in the partially denatured DsbC is 47 Å, which is markedly longer than 35 Å in the native protein. The much longer distance measured between the two N termini in a partially denatured molecule indicates that the unfolding of DsbC in the presence of GuHCl (1.5 M) renders the molecule more loose and flexible but not dissociated (Table 1). The very slow decay of the fluorescence anisotropy of the fully denatured DsbC could only arise from the rotation of the probe molecule together with the fluctuation of the conformation of DsbC rather than from DDEM processes. The interfluorophore distance in this case, which is far longer than the critical distance R_0 of BODIPY and could not be determined by DDEM measurements, implies the dissociation of the dimeric molecule in the fully denatured protein.

In summary, we have developed a valuable method, which consists of N-terminal-specific fluorescence labeling through a transamination reaction followed by DDEM measurements, to study the unfolding/folding processes of a dimeric protein. The transamination step provides a general approach for the selective attachment of a fluorophore to N-terminal amino acid residues, and the dimeric structure of DsbC allows the introduction of two identical fluorophores so that the DDEM method can be used to trace its unfolding behavior. This combined strategy is useful to investigate conformational changes of other dimeric proteins under variable conditions. An important development would be to combine the specific labeling method with DDEM measurement at the single-molecule level. Furthermore, this labeling approach could also be extended to nondimeric protein molecules and would therefore broaden the scope of application of fluorescence spectroscopy.

Experimental Section

General: DsbC (**1**) was prepared as reported previously^[7,9] from plasmid *pDsbC*, which contains the full-length DsbC precursor gene. Glyoxylate was purchased from Acros. BODIPY FL hydrazide was purchased from Molecular Probes, Inc. MALDI TOF mass spectrometry was performed on a Bruker BIFLEX III instrument.

3: DsbC (**1**; 1 mg) was dissolved in an aqueous solution of sodium acetate (2 mL; 1 M, pH 5.5) containing glyoxylate (0.1 M) and CuSO₄ (5 mM) and was stirred for 1 h at 296 K. The reaction was quenched through the addition of ethylenediaminetetraacetic acid diammonium salt to a final concentration of 20 mM followed by dialysis against sodium phosphate buffer (0.1 M, pH 7.4).

4: BODIPY FL hydrazide (**2**; 200 µL; 1.96 mM in MeOH) and concentrated HCl (to a final concentration of 0.5 M) were consecutively added to **3**, and the mixture was stirred for 1 h at 296 K in the dark. Sodium cyanoborohydride (5 equiv relative to the protein; Sigma) was then added and the solution was incubated overnight at 277 K. The mixture was applied onto a Sephadex G-25 column to remove any remaining free BODIPY dye and the excess reducing reagent. The protein fraction **4**, which displays an absorbance at both 280 and 505 nm, was collected and then thoroughly dialyzed against phosphate buffer. The efficiency of labeling was calculated from the

absorption spectra/molar absorptivities of the fluorescent probe **2** ($\epsilon = 80000 \text{ M}^{-1} \text{ cm}^{-1}$ at 505 nm)^[2] and the dimeric protein **1** ($\epsilon = 32340 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm).^[22] As a control, the same procedure was performed with DsbC in the absence of glyoxylate.

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