# Resolution of heterogeneous fluorescence into component decay-associated excitation spectra

## Application to subtilisins

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ABSTRACT Direct and indirect methods are described to combine steady-state and picosecond time-resolved fluorescence decay data to generate decay-associated excitation spectra. The heterogeneous fluorescence from a fluorophore mixture that models protein fluorescence was resolved into individual component excitation spectra. The two methods were also used to determine the excitation spectra associated with each of the decay time components for the proteins subtilisin Carlsberg and BPN'. On the basis of associated spectra, the decay components of both proteins were assigned to individual (or groups of) emitting species. The two approaches used to generate the decay-associated excitation spectra are compared and their general application to protein fluorescence studies is discussed.

## INTRODUCTION

Time-resolved fluorescence spectroscopy is a widely used method in investigations of the structure, function, and dynamics of biological systems (1). The method utilizes the sensitivity of the excited state of the fluorophore to its environment. However, the fluorescence emission from biological samples is frequently heterogeneous, complicating the interpretation. This heterogeneity can arise in a number of ways. For example, identical fluorophores may be present in more than one environment when the fluorophore, or the macromolecule to which it is attached, can exist in different conformational states. Further examples include the emission from multiple tryptophan-containing proteins where each residue experiences a unique environment, proteins that contain both tyrosine and tryptophan residues, and from extrinsic fluorophores nonspecifically bound to membranes, proteins, and nucleic acids. In addition to ground-state heterogeneity, excited-state reactions can further complicate the analysis.

In some cases it is possible to reduce the emission heterogeneity by careful choice of excitation and emission wavelengths or by selection of other parameters such as fluorescence lifetime and accessibility to quenchers. Several instrumental and data analysis methods of resolving heterogeneous fluorescence have been developed and include matrix rank analysis (2, 3), time-resolved emission spectra (4), fluorescence decay and other associated spectra (5–7), phase-sensitive detection (8), phase-resolved spectra (9, 10), linked-function analysis (11), and principal component self-modeling analysis (12, 13).

In time-resolved spectroscopy, decay-associated spectra (DAS) have proved to be of considerable value (6, 14–17). Steady-state and time-resolved data are combined to generate DAS, which represent the relative contributions of individual lifetime components, as a function of wavelength, to the total fluorescence. The information from DAS can be used to assign the lifetime to individual (or groups of) emitting species. A natural extension of this approach, proposed by Knutson et al. (6, 7), would be to similarly resolve the excitation spectrum of each lifetime component, generating decay-associated excitation spectra (EDAS). In cases where the fluorescence efficiency of each lifetime component is independent of excitation wavelength, individual EDAS will be proportional to the absorption spectrum of the corresponding lifetime component. To date only two brief applications of this method have been published (18, 19).

In this report three heterogeneous samples are analyzed and two methods of generating EDAS are considered. Initially a mixture of tyrosine and 1-methyltryptamine was examined to serve as a model for proteins emitting from both tyrosine and tryptophan residues (8). We also report the first EDAS measurements on proteins using subtilisins Carlsberg and BPN' as examples.

## MATERIALS AND METHODS

### Materials

L-Tyrosine (Sigma Chemical Co., St. Louis, MO) was recrystallized three times from water. 1-Methyl-3-(2-aminoethyl) indole, (1-methyl-

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tryptamine) was prepared according to Barton et al. (20). Crystalline subtilisins Carlsberg (Protease type VIII lots 87F-0490, 96F-0396, and 87F-0489) and BPN′ (Protease "Nagarase" type XXVII lots 97F-0218 and 117F-0656) were purchased from Sigma Chemical Co. Purification of the proteins by high performance liquid chromatography (HPLC) and the precautions taken to minimize autolysis have been described elsewhere (21).

## Spectroscopic measurements

Time-resolved fluorescence measurements were performed using the technique of time-correlated single photon counting with laser/microchannel plate-based instrumentation. The instrumentation and procedures used for time-resolved and steady-state measurements on the subtilisin proteins have been described previously (21). Time-resolved data were collected at 42.2 ps/channel for tyrosine and 1-methyltryptamine samples, over 1,024 channels. The spectral resolution used throughout this study was 4 nm. For all samples, corrections were made for the signal from the appropriate "buffer only" blank. All measurements were performed at 15°C.

## Data analysis

The function describing the fluorescence decay following $\delta$-function excitation is assumed to be a sum of exponentials:

$$I(\lambda_{em}, t) = \sum_i \alpha_i(\lambda_{em}) \exp(-t/\tau_i), \quad (1)$$

where $\tau_i$ is the decay time of the $i$th component and $\alpha_i(\lambda_{em})$ is its preexponential factor at emission wavelength $\lambda$. Because this model assumes $\tau_i$ is independent of $\lambda_{em}$, decay curves taken at different emission wavelengths can be analyzed simultaneously (22). Data were analyzed by a global least squares iterative convolution method. Adequacy of the exponential decay fitting was judged by inspection of the plots of weighted residuals and by the statistical parameters $\chi^2$, the reduced chi square (23), and SVR, the serial varience ratio (24). Once this model has been fit to the data, the emission spectra associated with each individual decay component can be derived (5) from:

$$I_i(\lambda_{em}) = I_{ss}(\lambda_{em}) \left[ \alpha_i(\lambda_{em})\tau_i \Big/ \left( \sum_i \alpha_i(\lambda_{em})\tau_i \right) \right], \quad (2)$$

where $I_i(\lambda_{em})$ is the emission spectrum associated with the $i$th component, $I_{ss}(\lambda_{em})$ is the total steady-state emission spectrum, and $\alpha_i(\lambda_{em})\tau_i / (\Sigma_i \alpha_i(\lambda_{em})\tau_i)$ is the fractional fluorescence of the $i$th component at emission wavelength $\lambda_{em}$. Similarly, if $\tau_i$ is independent of the excitation wavelength, $\lambda_{ex}$, within a given range, then $\lambda_{em}$ can be replaced by $\lambda_{ex}$ in Eq. 2. $I_i(\lambda_{ex})$ will then represent the excitation spectrum associated with the $i$th component (EDAS). In practice, to obtain EDAS directly, one measures decay curves at a fixed emission wavelength $\lambda'_{em}$ for a series of excitation wavelengths. These data are then analyzed simultaneously, with the constraint that the lifetimes are independent of excitation wavelength, to give the corresponding fractional fluorescences at each excitation wavelength for the decay components measured at $\lambda'_{em}$. The corrected steady-state excitation spectrum taken at the same spectral resolution and fixed emission wavelength as the time-resolved data is then recorded and EDAS are calculated according to the modified version of Eq. 2 ($\lambda_{em}$ replaced by $\lambda_{ex}$).

EDAS can also be determined "indirectly" (given the acronym IEDAS [indirect excitation DAS]; 7, 18, 19). It will be seen that the indirect method eliminates the requirement for a wavelength tunable excitation source in the fluorescence decay instrument. Consider Fig. 1,

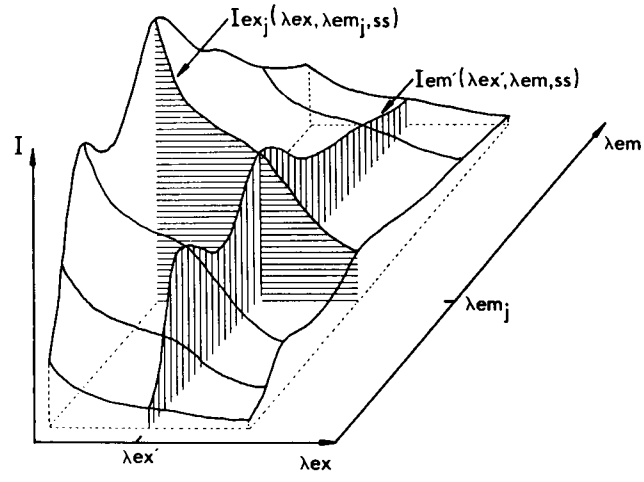


FIGURE 1 A representation of steady-state fluorescence intensity versus excitation and emission wavelengths.

a three dimensional surface of steady-state fluorescence intensity versus excitation and emission wavelengths, $I(\lambda_{ex}, \lambda_{em}, ss)$, which can be described by Eq. 3:

$$I(\lambda_{ex}, \lambda_{em}, ss) \propto \sum_{i=1}^{N} \epsilon_i(\lambda_{ex})\, \alpha_i(\lambda_{ex}, \lambda_{em})\, \tau_i, \quad (3)$$

where $N$ is the number of lifetime components and $\epsilon_i(\lambda_{ex})$ is the molecular absorptivity at $\lambda_{ex}$. The corresponding expression for a particular steady-state excitation spectrum, $I_{ex}j(\lambda_{ex}, \lambda_{em}j, ss)$, measured at emission wavelength $\lambda_{em}j$ is:

$$I_{ex}\, j(\lambda_{ex}, \lambda_{em}j, ss) = \sum_{i=1}^{N} \epsilon_i(\lambda_{ex})\, \tilde{\alpha}_i(\lambda'_{ex}, \lambda_{em}j)\, \tau_i, \quad (4)$$

where $\tilde{\alpha}_i(\lambda'_{ex}, \lambda_{em}j)$ is the preexponential factor normalized such that $\Sigma\tilde{\alpha}_i(\lambda'_{ex}, \lambda_{em}j)\tau_i$ represents the steady-state emission spectrum measured at the excitation wavelength $\lambda'_{ex}$. It follows from Eq. 4 that a series of steady-state excitation spectra $I_{ex}j(\lambda_{ex}, \lambda_{em}j, ss)$ recorded at particular emission wavelengths $\lambda_{em}j$, and fractional fluorescences measured at these same emission wavelengths (from a DAS experiment performed at excitation wavelength $\lambda'_{ex}$), can be related as in Eq. 5:

$$\begin{pmatrix} Iex_{1,1} & Iex_{1,2} & \cdots & Iex_{1,n} \\ Iex_{2,1} & Iex_{2,2} & \cdots & Iex_{2,n} \\ \vdots & \vdots & & \vdots \\ Iex_{m,1} & Iex_{m,2} & \cdots & Iex_{m,n} \end{pmatrix} = \begin{pmatrix} \epsilon_{1,1} & \epsilon_{1,2} & \cdots & \epsilon_{1,N} \\ \epsilon_{2,1} & \epsilon_{2,2} & \cdots & \epsilon_{2,N} \\ \vdots & \vdots & & \vdots \\ \epsilon_{m,1} & \epsilon_{m,2} & \cdots & \epsilon_{m,N} \end{pmatrix} \cdot \begin{pmatrix} M_{1,1} & M_{1,2} & \cdots & M_{1,n} \\ M_{2,1} & M_{2,2} & \cdots & M_{2,n} \\ \vdots & \vdots & & \vdots \\ M_{N,1} & M_{N,2} & \cdots & M_{N,n} \end{pmatrix}. \quad (5)$$

$Iex_{m,n}$ are the steady-state excitation spectral values over the set of emission wavelengths $\lambda_{em}j$, $j = 1, 2, \ldots n$ (at which excitation spectra

and fluorescence decay data are obtained) and over the set of excitation wavelengths $\lambda_{ex}k$, $k = 1, 2 \ldots m$ (of the measured excitation spectra). $M_{N,n}$ are the values of $\tilde{\alpha}_i(\lambda'_{ex}, \lambda_{em}j)\tau_i$ for $i = 1, 2, \ldots N$ and for $j = 1, 2, \ldots n$. The desired IEDAS are the set of $\epsilon_i(\lambda_{ex}l \rightarrow \lambda_{ex}m)$, i.e., the columns in the second matrix of Eq. 5. In our IEDAS program $n > N$ (the maximum values for $N$, $n$, and $k$ are presently 4, 25, and 150, respectively) and the IEDAS are overdetermined by a multiple linear least squares analysis. In the multiple least squares analysis the residuals are all assigned unit weights.

## RESULTS

To test the IEDAS program's ability to resolve the component excitation spectra of a heterogeneous sample, a mixture of L-tyrosine and 1-methyltryptamine was analyzed. This served as a model for proteins emitting from both tyrosine and tryptophan residues. Data from time-resolved measurements at an excitation wavelength of 290 nm were fitted with two decay times of 10.190 ± 0.006 and 3.340 ± 0.003 ns ($\chi^2 = 1.03$, SVR = 1.88) (Lifetimes are given ± their standard deviations. These errors are derived from the diagonal elements of the covariance matrix in the nonlinear least squares analysis.). These decay times correspond to the singlet lifetimes of 1-methyltryptamine and L-tyrosine, respectively, both of which exhibit single exponential decay kinetics. The time-resolved data, taken at 10 emission wavelengths ($\lambda_{ex}$ = 290 nm, $\lambda_{em}$ = 300–370 nm), were combined with 90 point excitation spectra at the same 10 emission wavelengths to derive the IEDAS. The IEDAS for 350-nm emission are shown in Fig. 2; the DAS and steady-state spectra of both the mixture and individual components are included for comparison. IEDAS derived for other emission wavelengths were also superimposeable with the corresponding normalized steady-state excitation spectra (not shown).

Decay-associated excitation spectra should assist in the assignment of decay components to individual (or groups of) emitting species. One such application is the resolution of tyrosine and tryptophan emission from proteins. Fig. 3 shows the decay-associated excitation spectra for subtilisin BPN′ at two emission wavelengths. The excitation spectra were resolved using both the direct and indirect methods. In the direct method (EDAS), time-resolved data taken at a fixed emission wavelength of 310 nm and 10 excitation wavelengths from 283 to 300 nm, analyzed simultaneously, required four decay components for an acceptable fit: 7.60 ± 0.02, 2.660 ± 0.006, 0.345 ± 0.005, and 0.086 ± 0.001 ns (global $\chi^2 = 1.09$, SVR = 1.76). These values were also used to model the data taken at an emission wavelength of 342 nm ($\chi^2 = 1.10$, SVR = 1.84). In the indirect method (IEDAS), 292 nm was used as the constant excitation wavelength in the time-resolved measurements. Data

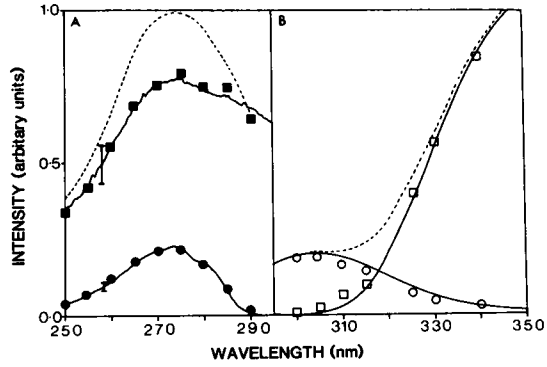


FIGURE 2 (*A*) IEDAS for a mixture of L-tyrosine and 1-methyltryptamine at an emission wavelength of 350 nm. The two components each had an absorbance of 0.05 at 290 nm and the buffer was 0.01 M cacodylate, pH 7. Measured steady-state excitation spectra ($\lambda_{em}$ = 350 nm) of the mixture (———) and of separate solutions of 1-methyltryptamine (■) and L-tyrosine (●). The solid lines running through the symbols are the derived IEDAS from the 10.19-ns (1-methyltryptamine) and 3.34-ns (L-tyrosine) lifetime components at an emission wavelength of 350 nm. The spectra were normalized to have the same value at 270 nm. For IEDAS curves in this and subsequent figures error bounds at one standard deviation were obtained from the multiple linear regression analysis; a representative error bar is shown for each component. (*B*) Normalized DAS for the sample described above. DAS of the 10.19-ns (□) and 3.34-ns (○) lifetime components. The solid lines running through the symbols are measured steady-state emission spectra ($\lambda_{ex}$ = 290 nm). The steady-state emission spectrum of the mixture is also included (————). For DAS, propagated errors computed from the standard errors of the fractional fluorescences are within the contours of the plotted symbols.

from 10 emission wavelengths were fitted with the same lifetime values as used in the computation of EDAS ($\chi^2 = 1.12$, SVR = 1.71).

Decay-associated excitation spectra for subtilisin BPN′ generated by EDAS or IEDAS are consistent (Fig. 3). At an emission wavelength of 310 nm, the 7.60-ns component has an associated tryptophan-like excitation spectrum, while that of the more intense 2.660-ns component resembled the tyrosine excitation spectrum. The spectra associated with the other two components were too weak to assign, and in the case of IEDAS were poorly defined. When 342 nm was chosen as the emission wavelength, the tryptophan-like 7.60-ns component predominated. While the other three components were weak, the 2.660-ns component was sufficiently well-defined by IEDAS to assign to tyrosine.

It is important to note that attempts to generate the IEDAS for subtilisin BPN′ using excitation constant wavelengths ($\lambda'_{ex}$) of 295 or 300 nm failed, even though the fits to the time-resolved data were good. The spectra that resulted were not consistent with EDAS and were characterized by a significant negative component spectrum. Fig. 4 shows the decay-associated excitation spec-

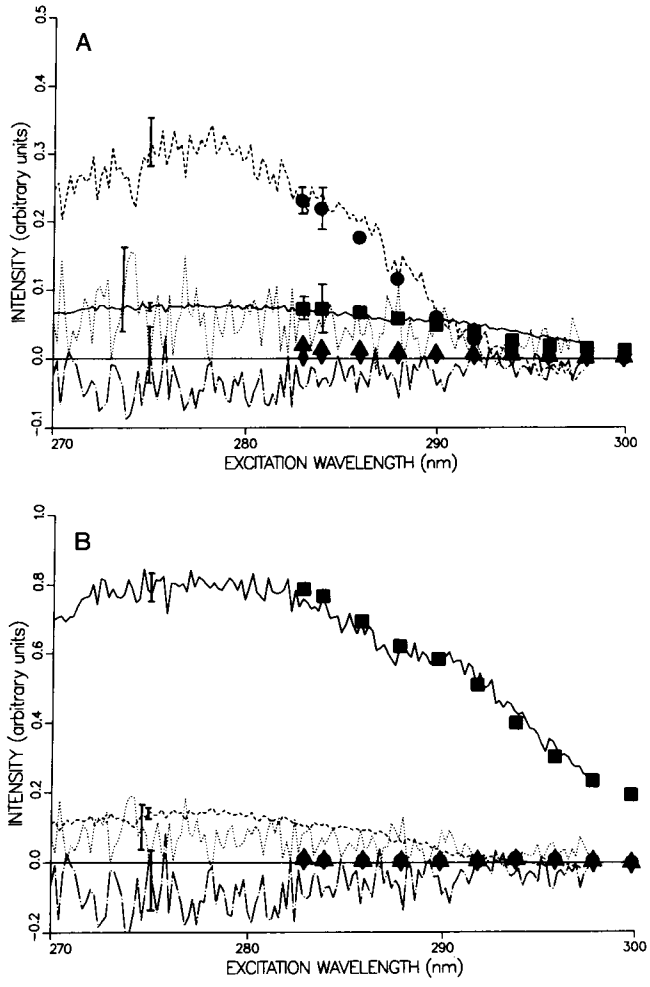


FIGURE 3 Decay-associated excitation spectra for subtilisin BPN′ at emission wavelengths of (*A*) 310 nm and (*B*) 342 nm. IEDAS of the 7.60-ns (—), 2.66-ns (— — —), 0.34-ns (· · ·), and 0.09-ns (— · —) components. EDAS of the 7.60-ns (■), 2.66-ns (●), 0.34-ns (▲), and 0.09-ns (♦) components. EDAS were normalized to IEDAS at 288 nm. For EDAS, error bars are propagated errors computed from the standard errors of the fractional fluorescences. When no error bars are given, errors are within the contours of the plotted symbols. For IEDAS, $\lambda'_{ex}$ was 292 nm, 10 emission wavelengths were used (306–345 nm), and each steady-state excitation spectrum contained 140 points. The protein samples were purified by HPLC and typically had an absorbance of 0.08 at the excitation wavelength. The buffer was 10 mM phosphate (pH 6.2) containing 63 mM NaCl, and the temperature was 15°C.

tra for subtilisin Carlsberg at an emission wavelength of 320 nm. The time-resolved data used to generate the EDAS, taken at 10 excitation wavelengths (283–300 nm) and an emission wavelength of 320 nm, were fitted with three components of lifetimes 3.278 ± 0.002, 0.247 ± 0.003, and 0.081 ± 0.001 ns ($\chi^2$ = 1.11, SVR = 1.78). For IEDAS of subtilisin Carlsberg the excitation constant wavelength was 295 nm and the time-resolved data were taken from a previous study (21). The IEDAS lifetime

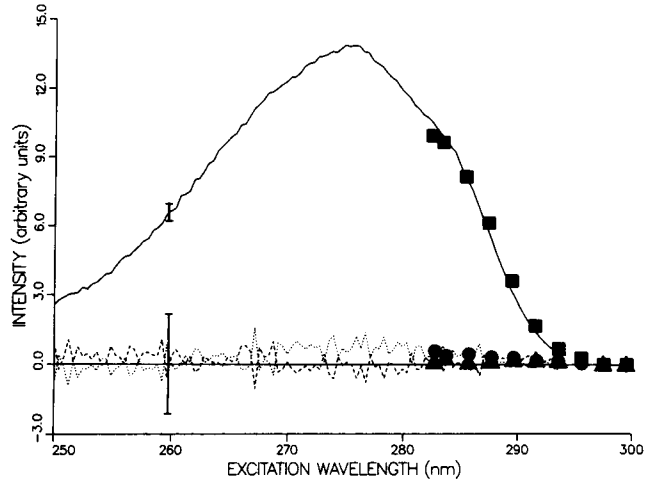


FIGURE 4 Decay-associated excitation spectra for subtilisin Carlsberg at an emission wavelength of 320 nm. IEDAS of the 3.34-ns (—), 0.20-ns (— — —), and 0.07-ns (· · ·) components. EDAS of the 3.28-ns (■), 0.25-ns (●), and 0.08-ns (▲) components. EDAS were normalized to IEDAS at 286 nm. For IEDAS $\lambda'_{ex}$ was 295 nm, nine emission wavelengths were used (300–365 nm), and each steady-state excitation spectrum contained 112 points.

values (3.338, 0.197, and 0.069 ns) were similar to those used in the EDAS analysis, particularly in the case of the dominant long-lifetime component, and as expected the EDAS and IEDAS results were in close agreement. The excitation spectrum associated with the long-lifetime component (Fig. 4) was clearly tyrosine-like. The two short-lifetime components make such a small contribution that it was not possible to unambiguously assign them. An attempt to generate IEDAS for subtilisin Carlsberg, using 300 nm as the excitation constant wavelength, was unsuccessful, giving results similar to those described for subtilisin BPN′ at $\lambda'_{ex}$ = 295 and 300 nm.

## DISCUSSION

The association of a spectral distribution with a particular decay component provides information on both the identity and environment of an individual (or group of) emitting species. In this study we are concerned with the resolution of the excitation spectra of proteins exhibiting heterogeneous fluorescence into component decay-associated excitation spectra. To model the heterogeneity that can arise in protein fluorescence as a result of emission from both tyrosine and tryptophan residues, we studied a mixture of L-tyrosine and 1-methyltryptamine. In the model system we were able to compare the measured excitation and emission spectra of solutions of the pure components with the spectra derived by applying the

IEDAS method to the mixture. As illustrated in Fig. 2, the derived IEDAS were superimposable with the measured pure component excitation spectra. For a two-component model system the minimum requirement for resolution of IEDAS are two time-resolved measurements at an excitation wavelength where both components contribute to the emission, as well as two corrected steady-state excitation spectra measured at the same pair of emission wavelengths as the time-resolved data. However, overdetermination is advisable and preferred, particularly in those cases where accurate resolution of the decay times is difficult.

Subtilisins are serine proteases produced by various species of *Bacillus*. Subtilisin Carlsberg (from *Bacillus licheniformis*) and subtilisin BPN′ (from *Bacillus amyloliquefaciens*) have been extensively studied (25) and their crystal structures are known at high resolution (26, 27). Subtilisin Carlsberg contains 274 amino acid residues and shows a high degree of structure and sequence homology with subtilisin BPN′, differing at only 84 positions and by a deletion at $Pro^{56}$ of BPN′. Subtilisin Carlsberg contains one tryptophan ($Trp^{113}$) and 13 tyrosine residues, whereas subtilisin BPN′ contains 3 tryptophan residues ($Trp^{113}$, $Trp^{106}$, and $Trp^{241}$) and 10 tyrosine residues (25). Subtilisin BPN′ emission is dominated by tryptophan; however, subtilisin Carlsberg is an exception to the general rule that in proteins that contain both tyrosine and tryptophan residues, the overwhelming contribution to the emission is from tryptophan (28).

In a previous study (21), we modeled the emission from subtilisin BPN′ excited at 295 or 300 nm with four decay components. On the basis of DAS the 8.00-, 0.25-, and 0.05-ns components were assigned to tryptophan emission and we suggested that the 2.45-ns component, while predominantly tryptophan-like, may contain a contribution from tyrosine at short emission wavelengths. The decay-associated excitation spectra for subtilisin BPN′ generated by both EDAS and IEDAS are shown in Fig. 3. Global analysis of the time-resolved data required to generate the decay-associated excitation spectra (IEDAS; $\lambda_{ex}$ 292 nm, $\lambda_{em}$ 306–345 nm: EDAS; $\lambda_{ex}$ 283–300 nm, $\lambda_{em}$ 310 and 342 nm) resulted in decay times of 7.60, 2.66, 0.34, and 0.08 ns. The comparable decay times reported previously (see above) are required to accurately model the data when 295 or 300 nm excitation is used ($\lambda_{em}$ 310–430 nm) because at these wavelengths the tyrosine contribution is very small and poorly defined. In order to enhance any tyrosine contribution relative to tryptophan, the decay-associated spectra were derived for an emission wavelength of 310 nm. The 2.66-ns component clearly displays a tyrosine-like excitation spectrum, while as expected, the 7.60-ns component spectrum resembles that of tryptophan. At an emission wavelength of 342 nm, the tryptophan contribution is enhanced and the 7.60-ns component predominates. A weak tyrosine-like excitation spectrum is resolved by IEDAS for the 2.66-ns component. At both emission wavelengths the 0.34- and 0.08-ns components were too weak to assign. In the decay-associated excitation spectra for subtilisin Carlsberg at an emission wavelength of 320 nm, the tyrosyl origin of the 3.34-ns lifetime component is apparent, as is the highly quenched tryptophan contribution.

The results for the subtilisins discussed above demonstrate the utility of decay-associated excitation spectra in assigning lifetime components to individual, or groups of, emitting species. They concur with the assignments we made previously on the basis of DAS (21), and in the case of subtilisin BPN′ confirm our suggestion that the 2.45-ns component contains contributions from both tryptophan and tyrosine (the former predominating at excitation wavelengths >295 nm). The measurements on the subtilisins also illustrate some of the practical considerations in the application and interpretation of the analogous EDAS and IEDAS approaches. In EDAS, time-resolved measurements are performed at a series of excitation wavelengths while the emission wavelength is held constant. This of course requires a tuneable excitation source. With our laser-based instrument the effective excitation range for proteins was 283–305 nm, limited by the output of the rhodamine 6G dye laser and protein absorptivity. Fortunately, this is not a serious limitation because the absorption spectra of tyrosine and tryptophan are significantly different in this region. In practice, however, the laser and detection optics had to be reoptimized each time the excitation wavelength was changed. The excitation wavelength range can be further extended by the use of other dyes. The IEDAS method has the advantage that the time-resolved measurements are performed at a fixed excitation wavelength. A further practical advantage of IEDAS is that decay-associated excitation spectra can be generated for any of the emission wavelengths used in the construction of the required DAS data set, without making further measurements.

The choice of the excitation constant wavelength for IEDAS is important (7). For example, IEDAS of subtilisins Carlsberg and BPN′ failed when the excitation constant wavelength was 300 nm. IEDAS of subtilisin BPN′ also failed when the time-resolved data were collected at 295 nm excitation. In these cases, the steady-state excitation spectra contained information on the tyrosine contribution but the time-resolved data did not. An excitation constant wavelength for time-resolved measurements must be chosen that excites all the species contributing to the steady-state excitation spectra. IEDAS cannot be used if any two of the excitation spectra have the same shape. If $\Sigma_{i=1}^{N} a_i \epsilon_{m,N} = 0$ when not all the $a_i$'s are zero, then the matrix inversion will fail in the least squares analysis. When spectra are closely similar in

shape the matrix inversion becomes badly conditioned, consequently the results are unreliable and very sensitive to the "noise" on the data. The EDAS method has an important advantage over IEDAS in that the validity of the assumption that the lifetimes are independent of the excitation wavelengths is tested directly by the global $\chi^2$. While IEDAS measurements may be more convenient because of the pitfalls, it is wise to corroborate a portion of the IEDAS results using the EDAS method.

Resolution of decay-associated excitation spectra is limited by the ability to accurately resolve decay times. In the protein examples used in this study, we are attempting to describe the decay kinetics of up to 13 tyrosine residues with a single component. That we have had any success at all is a reflection of the homogeneity of the tyrosine emission. The value of the "tyrosine" lifetime (an intensity weighted average), and its relative contribution, will vary over the fluorescence intensity surface. EDAS and IEDAS sample different regions of this surface, which accounts for the minor variation in the recovered lifetimes and spectral resolution. In addition to ground-state heterogeneity, multiexponential decay kinetics can also result from excited-state reaction. In this case, the recovered decay times are characteristic of the system and are not associated with specific emitting species. It is possible in favorable cases to recover the spectra associated with specific emitting species (7, 29). Knutson et al. (6) have also pointed out that if only one ground-state species is excited the EDAS will be identical for all lifetime components.

The effect of tyrosine to tryptophan energy transfer is another important consideration in the application of EDAS and IEDAS to proteins. Where energy transfer is not significant, tyrosine and "pure" tryptophan excitation and emission spectra will be observed in EDAS and DAS, respectively. This appears to be the case with subtilisins Carlsberg and BPN'. If energy transfer is complete, individual EDAS will represent the sum of a tryptophan lifetime component associated excitation spectrum, and the excitation spectra of any tyrosine residues that transfer to that component. No direct tyrosine emission spectral components will be observed in the DAS. In proteins where a significant fraction of the tyrosine residues partially transfer their energy to tryptophan, the decay kinetics of both the donors and acceptors are expected to be nonexponential (30) and both EDAS and IEDAS will fail.

In addition to the examples given, we expect decay-associated excitation spectra to have applications in many situations where heterogeneous fluorescence is observed. For example, IEDAS has recently been applied to the resolution of the excitation spectra of ground-state rotamer populations in fluorescent cholesterol derivatives (31). This approach can be extended since, in addition to the time axis, excitation spectra can also be associated with rotational decay rates or quencher concentration (6, 7).

In the cases where a model that assumes a continuous distribution of exponential decays is appropriate, it is possible to approximate such a model with a small number of discrete components and apply the formulations we have developed above for the determination of IEDAS.

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