Global Resolution of Heterogeneous Decay by Phase/Modulation Fluorometry: Mixtures and Proteins†

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ABSTRACT: Fluorescence lifetime data obtained by the phase/modulation technique are used to resolve the heterogeneous fluorescence decays of model mixtures and of horse liver alcohol dehydrogenase. This was made possible by a global nonlinear least-squares procedure for simultaneous analysis of multiple data sets. Analyses of simulated data suggest that biexponential lifetimes that differ by as little as 20% may be resolvable by this method.

Fluorescence lifetime measurements have been valuable in studies of the static and dynamic structure of proteins, nucleic acids, and membranes. In biological systems monoexponential decay is rare. Deviations from monoexponential decay kinetics may have their origin in sample heterogeneity or in a variety of excited-state reactions that take place on the same time scale (nanoseconds) as fluorescence decay. The ability to resolve these complex decays allows attention to be focused, for example, on a particular tryptophan residue in a protein (Ross et al., 1981; Eftink & Jameson, 1982) or on fluorescence probes located in particular domains of a biological membrane (Matayoshi & Kleinfeld, 1981; Lentz et al., 1982). Similarly, a detailed decomposition of excited-state processes (such as energy transfer, excited-state proton transfer, or exciplex formation) provides knowledge regarding spatial and dynamic changes in biological macromolecules.

Both pulse and phase/modulation fluorometry have found wide use in the biochemical laboratory. Pulse methods, including time-correlated monophoton counting, provide a decay curve in real time convolved with a lamp profile of finite width. There is a large literature describing numerical methods for analyzing real-time decay data [for review, see Badea & Brand (1979)]. Phase/modulation provides a Fourier transform of the real-time response. There are several methods available for analyzing phase/modulation data in terms of multiexponential decay. These include the algebraic formula of Weber (1981) and the nonlinear least-squares approaches of Matayoshi & Kleinfeld (1981) and Jameson et al. (1984). Phase instruments with multiple frequency excitation have further enhanced the capability of this technique (Gratton et al., 1984).

The usual approach for analyzing a photophysical system has been to measure the fluorescence decay under a variety of experimental conditions. Each data set is then analyzed individually to obtain parameters such as the exponential decay constants and amplitudes. Graphical analysis of the decay parameters as a function of some other variable is then used to test a proposed model for the overall fluorescence decay. For example, in the case of excited-site proton transfer (Laws & Brand, 1979) changes of decay parameters as a function of emission wavelength and pH have been utilized to study the mechanisms involved and to obtain values for the rate constants of the excited state.

We have recently pointed out (Beechem et al., 1983; Knutson et al., 1983) that there is a considerable advantage in analyzing several decay curves at the same time (global analysis) in cases where decay sets have a parameter in common or linkage between parameters. For example, by changing wavelength, one may often obtain a series of decay measurements that contain different fractional amplitudes of the same lifetimes. A similar approach has been applied to other kinds of data (Johnson et al., 1981; Ackers et al., 1975). Global analysis of pulse fluorometric data provided order of magnitude improvement in resolving power. We show here that global analysis is readily adaptable to phase/modulation spectroscopy.

Materials and Methods

Fluorescence phase/modulation lifetimes and spectra were measured with an SLM 4800 nanosecond fluorometer (SLM/Aminco, Urbana, IL). Horse liver alcohol dehydrogenase (HLADH,[1] EC 1.1.1.1) was obtained from Boehringer Mannheim as a crystalline suspension and prepared for use as previously described (Ross et al., 1981). Pure anthracene (ANTH) was a gift from Professor R. P. DeToma. *N*-Acetyltryptophanamide (NATA) was purchased from Vega Biochemicals (Tucson, AZ), and 9-cyanoanthracene (9CNA) was an Aldrich (Milwaukee, WI) product. Other chemicals were reagent grade. The final concentrations used in this study were 2×10^{-6} M 9CNA and 10^{-5} M ANTH. NATA was used as a fluorescent standard at 10^{-4} M in 0.05 M sodium phosphate buffer, pH 7.4. HLADH was used at a concentration of 10^{-5} M in sodium phosphate buffer, pH 7.4.

Apparent phase and modulation lifetimes were obtained by using standard data collection routines supplied with the instrument, taking advantage of monoexponential standards to compensate for wavelength dependence of transit time in the photomultiplier (Lakowicz & Cherek, 1980; Barrow & Lentz, 1983). For the mixed dye systems, reference was made to pure anthracene solutions, while HLADH experiments used NATA as the "color shift" standard.

Theory

The response of a luminescence system to sinusoidally modulated excitation is predicted by means of the Fourier transform of the impulse response. The modeling of phase/modulation data can be carried out with the same adjustable

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[1] Abbreviations: HLADH, horse liver alcohol dehydrogenase; ANTH, anthracene; NATA, *N*-acetyltryptophanamide; 9CNA, 9-cyanoanthracene.

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parameters used in real-time decay studies. To evaluate performance of a model involving data obtained under differing conditions (excitation or emission wavelength, frequency, pH, concentration, etc.), global parameter mapping is of value. A global map links the various measured responses with the specific fitting function for that decay set. The fitting function used may have one or more parameters in common with functions appropriate to other decay sets. Determination of a single decay parameter can thus be influenced by data from different experiments. A general mapping matrix approach was developed for global analysis of pulse decays (Knutson et al., 1983) and may be adapted to phase/modulation data.

Consider a biexponentially decaying system with closely spaced lifetimes. Further, let the component spectra be different but strongly overlapped. In this case, variation of emission wavelength provides data that contain different fractions of the lifetime components; the lifetimes, however, are invariant.

For a system monitored at n wavelengths, there will be $n + 2$ global parameters, corresponding to the two lifetimes τ_1, τ_2, and n amplitudes (α_{11}, α_{12}, ..., α_{1n}). The corresponding second amplitudes are found by $\alpha_{2j} = 1 - \alpha_{1j}$. These lifetimes and amplitudes are linked to impulse response functions of the form:

$$I_j(t) = \alpha_{1j}e^{-t/\tau_1} + \alpha_{2j}e^{-t/\tau_2} \quad (1)$$

for the biexponential case. Thus, index j relates amplitude fractions α_{1j} and α_{2j} to emission lifetimes τ_1 and τ_2 at each wavelength j.

The functions describing phase/modulation data are simple transforms of the impulse responses in eq 1. For example, when the data are τ_p and τ_m (the apparent phase and modulation lifetimes, respectively), the fitting functions are

$$\tau_p = \frac{1}{\omega_{ij}}\left(\sum_k S_k / \sum_k C_k\right) \quad (2)$$

$$\tau_m = \frac{1}{\omega_{ij}}\left[\frac{I^2}{(\sum S_k)^2 + (\sum C_k)^2} - 1\right]^{1/2} \quad (3)$$

where

$$S_k = \alpha_{kj}\omega_{ij}/(1/\tau_k^2 + \omega_{ij}^2)$$

$$C_k = \alpha_{kj}/[\tau_k(1/\tau_k^2 + \omega_{ij}^2)]$$

$$\omega_{ij} = \text{angular frequency}$$

and $I = \sum_k \alpha_{kj}\tau_k$ is the total intensity. The nonlinear least-squares analysis was with respect to τ_p and τ_m, an approach used by Klausner et al. (1980). Jameson & Gratton (1983) have suggested that a direct fit to the phase angle and modulation will be advantageous in certain cases. The global algorithm used can be easily modified to do this.

Values of the global parameter set were determined by nonlinear least-squares analysis of all data, using relationships in eq 2 and 3. The optimization program employed a modified Gauss–Newton procedure (Johnson et al., 1976; Turner et al., 1981). The numerical determination of partial derivatives utilized in this program provides for rapid modification and testing of global models, since laborious development of closed analytical forms is bypassed. Following the determination of the best estimates for each parameter, the program computes confidence limits for simultaneously determined parameters, based on a 65% joint confidence region. This corresponds approximately to one standard deviation. Further tests of "goodness of fit" include examination for cross-correlation between parameters and random character in residuals. Global analysis can be readily implemented with many of the widely distributed nonlinear least-squares programs found in computing centers.

Table I: Typical Global Analyses on Simulated (Noisy) Phase/Modulation Data[d]

	simulation				
	I	II	III	IV	V
% noise levels (ps)	2 (100)	2 (100)	1 (50)	1 (60)	1 (60)
1 input	4	5	5	3	3
1 recovered	3.96	5.10	5.06	3.00	3.72
+/−	0.65	0.21	0.16	0.21	0.23
2 input	6	6	6	6	6
2 recovered	5.97	6.97	6.34	5.54	7.53
+/−	0.58	0.59	0.28	0.69	1.05
3 input				12	12
3 recovered				11.52	12.18
+/−				0.21	0.26
	a	*a*	*a*	*b*	*c*

[a] Frequencies, 30 and 10 MHz; preexponential mixtures, 0.75:0.25 and 0.25:0.75. [b] Frequencies, 30 and 10 MHz; mixtures, 0.1:0.3:0.6, 0.3:0.5:0.2, and 0.5:0.1:0.4. [c] Frequencies, 30, 18, and 6 MHz; mixtures as in footnote *b*. [d] All lifetimes in nanoseconds.

Results

The performance of global nonlinear least-squares analysis on phase/modulation data was evaluated via tests on known biexponential systems. Both synthetic data from a simulation program (providing an adjustable noise level) and experimental data were examined.

The synthetic biexponential systems and recovered parameters are listed in Table I. The first example, a biexponential doublet of 4.0 and 6.0 ns, was treated to produce 2% noise levels in τ_p and τ_m. All simulations were carried out with a single pair of admixtures, $\alpha_{11} = 0.75$ and $\alpha_{12} = 0.25$. Global analyses gave excellent results, returning accurate lifetime parameters. Analyses of the same data by algebraic heterogeneity or conventional (single-wavelength) nonlinear least squares did not return consistent or reasonable lifetimes (data not shown). The global procedure barely resolves a 5.0- and 6.0-ns doublet at 1% noise level. Global analysis could not resolve this close biexponential when the noise exceeded 1%.

Table I also contains typical results from analyses on ternary systems. An ability to resolve multiexponentials can be demonstrated for any method if lifetimes are well separated. Thus, these simulations were focused on closely spaced systems using a mutual ratio of 2. Weber (1981) pointed out the difficulty of resolving such a system with algebraic inversion; noise levels of 5 ps and less seem necessary. The ternary systems simulated here were resolved in spite of 1% ($\gtrsim$50 ps) noise. The success of global analysis will, of course, depend on several factors besides noise level. The relationships between lifetimes and amplitudes have obvious impact. Less obvious, yet important effects may arise from relationships between particular lifetimes and amplitudes. Similar considerations hold for choices of frequency and other instrumental settings, which were not exhaustively optimized in this study. A series of moderately difficult cases were chosen to demonstrate improved analytic capability on "typically" mixed systems.

To test the global method on real data, we first examined mixtures of two monoexponentially decaying dyes (ANTH and 9CNA) using two frequencies (30 and 18 MHz) and three emission wavelengths (410, 420, 430 nm). It is clear that decay measurements at these emission wavelengths (Figure

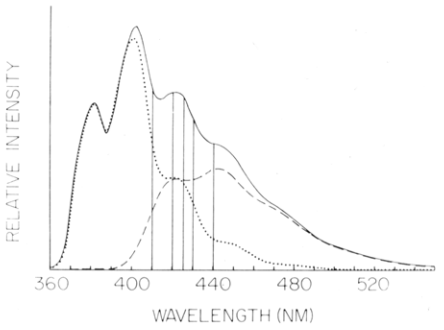

FIGURE 1: Technical emission spectrum of the 9CNA/ANTH mixture used. The optical configuration was the same as in Figure 2. Superimposed are the spectra of 9CNA (– – –) and ANTH (···). Solid vertical lines represent emission wavelengths used for global analyses. Spectra were normalized to show the relative contributions at each wavelength.

1) each contain different proportions of the two decay times. Figure 2 (right) shows the results of algebraic analyses. All values are reasonably close to the actual decay times. Figure 2 (center) shows the results of conventional nonlinear least-squares analyses on the mixture (analysis on individual data sets). Again, the values are reasonable. Global analysis of the entire data matrix for a biexponential yielded lifetimes in excellent agreement with the two unmixed dyes (Figure 2, left). (Pulse and phase fluorometric measurements on the unmixed dye solutions yielded identical results.) Actually, simultaneous analysis of data at three emission wavelengths but only one frequency (18 MHz) was sufficient to accurately determine the lifetimes in this system.

To generate a more difficult test case, a dynamic quencher (0.005 M KI) was added. The individually measured lifetimes became 3.9 and 7.2 ns for ANTH and 9CNA, respectively. Data at the five emission wavelengths shown in Figure 1 (each at two frequencies) were obtained. Figure 3 (right) shows the results of algebraic analyses. Obviously, this method did not resolve the doublet. Conventional nonlinear least-squares analyses (Figure 3, center) also failed to recover the correct lifetimes. Global analysis, however, yields lifetime parameters that agree with the unmixed quenched dyes (Figure 3, left).

It should be recognized that none of the emission wavelengths were in a resolved portion of the spectrum. It has previously been pointed out (Lakowicz & Cherek, 1981) that spectral separation of a species allows one to substitute its

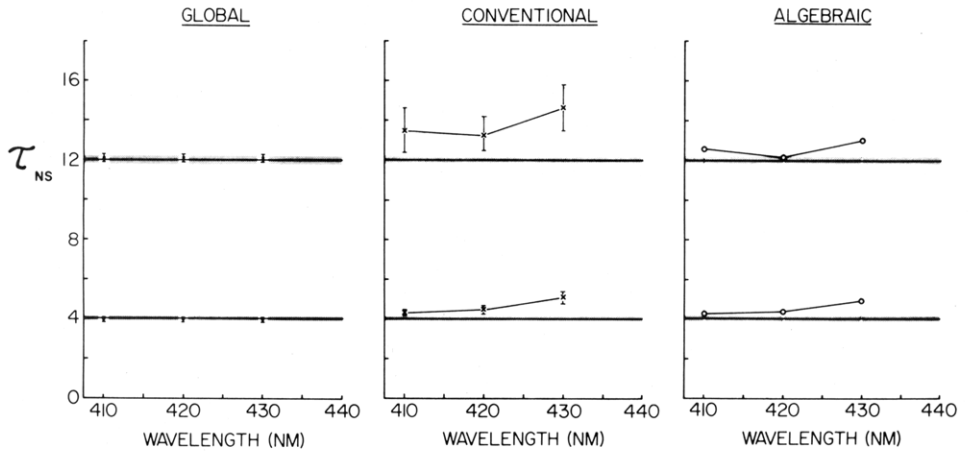

FIGURE 2: Recovered lifetimes vs. emission wavelength obtained by analyses of a mixture of 9CNA and ANTH in methanol. Modulation frequencies were 18 and 30 MHz, the excitation wavelength was 340 nm, and the emission band-pass was 16 nm. Individual component lifetimes, obtained from measurements on the unmixed dyes, are represented by the solid lines. The shaded region corresponds to the standard deviation of these unmixed data. The error bars about each point represent the standard error in the lifetimes. For least-squares analyses, they were estimated by monitoring χ^2 during directed searches in parameter space. For the algebraic solution, Jameson & Weber (1981) predict an uncertainty in the derived lifetimes that is 5–15-fold greater than the instrumental uncertainties in τ_p and τ_m. The 100–200-ps instrumental errors we observe would therefore correspond to 0.5–3.0-ns lifetime uncertainties.

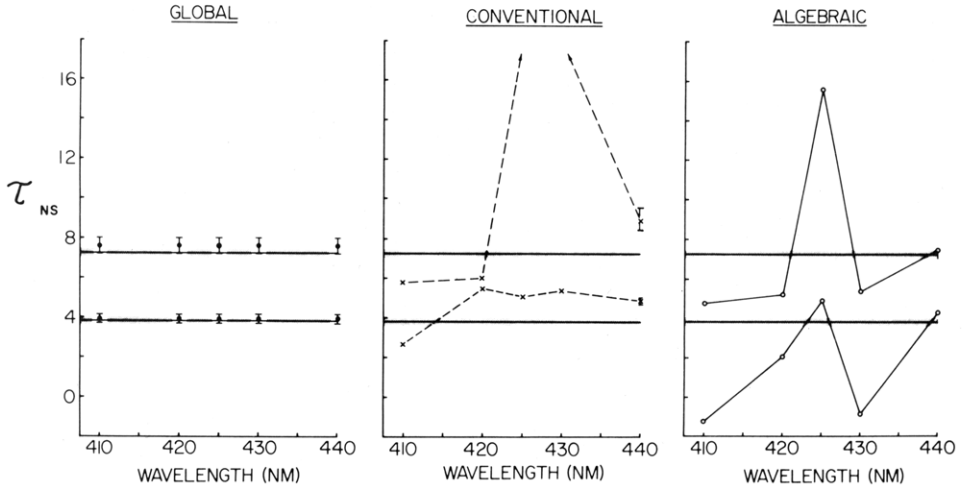

FIGURE 3: Recovered lifetimes vs. emission wavelength obtained by analyses on a quenched mixture (0.005 M KI) of ANTH and 9CNA in methanol. The optical configuration was the same as that in Figure 2. Again, the measured lifetimes of the unmixed quenched dyes are shown as solid lines, and the shaded areas represent the standard deviation in these component lifetimes. Error bars are obtained in the same way as in Figure 2. To facilitate presentation, however, only a single typical pair is presented.

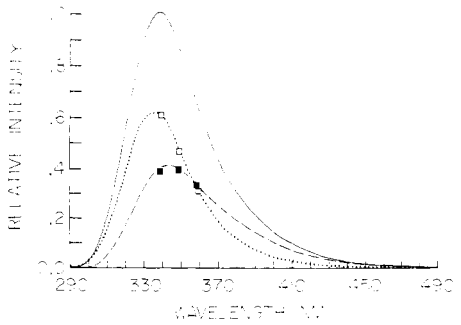

FIGURE 4: Steady-state contributions and decay-associated spectra (DAS) for the emission of HLADH in 0.05 M phosphate buffer, pH 7.4 [DAS from Knutson et al. (1982)]. The solid line represents total intensity. Dotted lines represent the spectrum associated with the 3.8-ns component and dashed lines the spectrum associated with the 7.2-ns component. These DAS were normalized with an 8% correction factor to compensate for the differences in excitation band-pass between phase and pulse instruments. The excitation wavelength was 295 nm. Modulation frequencies were 18 and 30 MHz. The open squares represent the steady-state contribution of the 3.8-ns component, using amplitudes recovered from the global analysis. The solid squares represent contributions of the 7.2-ns component.

lifetime into analyses at other wavelengths. One does not require complete (or even moderate) spectral resolution to accomplish global overdeterminations.

Next, we chose to examine the decay of HLADH, a system that was previously investigated by both pulse (Ross et al., 1981) and phase (Eftink & Jameson, 1982) methods. Pulse decay resolved the emission into two components of 3.8 and 7.2 ns. These lifetime components have been associated with two strongly overlapped but shifted spectra (Knutson et al., 1982). Ross et al. (1981) have shown that the longer lifetime can be selectively quenched with KI. The selective quenching of this component by acrylamide made it possible for Eftink & Jameson (1982) to obtain the same two lifetimes from phase/modulation data. As is shown in Figure 4, the lifetimes in this system can be obtained *directly* by global analysis of unquenched phase/modulation data. Figure 4 shows that observations of the fluorescence emission at 340, 350, and 360 nm result in different fractional amplitudes of two lifetimes. Global analysis of data at these wavelengths recovered lifetime values and fractional amplitudes in good agreement with those obtained by pulse fluorometry, as shown in Table II.

Discussion

The emission kinetics of biological systems are generally complex, reflecting both physical and chemical aspects of the interaction between a fluorophore and its local environment. To realize the full potential of nanosecond fluorometry in the investigation of biological molecules, it is essential to be able to determine the decay functions of the reporter group. To demonstrate this point, we used phase/modulation fluorometry to resolve emission from HLADH. This resolution required an ability to accurately determine exponential decay constants and preexponential terms.

This study employed unweighted nonlinear least-squares analysis. The agreement between phase global determined lifetimes of the mixture, the phase measurements of the individual dyes, and corresponding individual pulse determinations demonstrates the adequacy of unitary weighting in this application. Theoretically or empirically founded weighting functions (Bevington, 1969; Draper & Smith, 1966) would probably extend the useful range of applications for phase global analysis.

The global examples in this brief report have wide applicability in the analysis of excited-state heterogeneity. Multiple

Table II: Lifetimes of HLADH Fluorescence Recovered by Global Analysis As Compared with Published Values

T (°C)	τ_1 (ns)	τ_2 (ns)	condi-tions	source
20	3.95 ± 0.38	7.45 ± 0.54	*a*	this work, phase globals
18.5	3.75	7.09	*b*	this work, pulse verifications
20	3.6 ± 0.2	6.9 ± 0.4		Eftink & Jameson (1982)
27.5	3.8	7.0	*c*	Ross et al. (1981)
10	3.9	6.92	*b*	Ross et al. (1981)
10	3.8	6.72	*b*	Knutson et al. (1982)

[a] Excitation polarization vertical, emission at "magic angle" (Badea & Brand, 1979). [b] Excitation depolarized, emission at conjugate magic angle. [c] From corrected sum curves, parallel plus two perpendicular.

lifetimes recovered may be linked to spectra via application of the DAS process (Knutson et al., 1982) to either time-resolved emission spectra (Badea & Brand, 1979) or phase-sensitive spectra (Lakowicz & Cherek, 1981). Decay-associated spectra provide meaningful signatures whether the heterogeneity originates in the ground state or arises after excitation (Davenport et al., 1983) and may be obtained from ternary or more complex mixtures.

Global analysis is not limited in scope to simple linkage of lifetimes. The only requirement for overdetermination is a systemwide model that can be tested on multidimensional data. Experimental conditions may often be "tailored" to provide more data in those regions that strongly distinguish competing models once a global framework is established.

Fluorescence decay data, whether obtained by pulse or by phase/modulation methods, have usually been analyzed one decay curve at a time. This approach fails to exploit relationships that may exist among data sets. We suggest that nanosecond decay experiments be planned with data analysis in mind. It will often be possible to obtain a family of related data sets. In this way, complex decay constants may be resolved that would otherwise be unapproachable.

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Registry No. Alcohol dehydrogenase, 9031-72-5; anthracene, 120-12-7; 9-cyanoanthracene, 1210-12-4.

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Use of Radiolabeled Monofluoromethyl-Dopa To Define the Subunit Structure of Human L-Dopa Decarboxylase†

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ABSTRACT: Human L-Dopa decarboxylase (L-aromatic amino acid decarboxylase, DDC) has been purified from pheochromocytoma tissue, a benign tumor of the catecholamine-synthesizing cells of the adrenal medulla. The binding characteristics of a new radiolabeled enzyme-activated suicide inhibitor of DDC ([^{3}H]monofluoromethyl-Dopa, [^{3}H]MFMD) have been established, and the covalent linkage of the inhibitor to the enzyme has been used to identify that human DDC exists as a dimer of a 50-kDa subunit. An antibody to human DDC identically precipitates the enzyme activity from different human, rat, and mouse tissues. Our data demonstrate the value of [^{3}H]MFMD for probing the structure of DDC and facilitating the purification of this enzyme, and further emphasize the high degree of conservation of the DDC molecule over a wide variety of species.

This paper describes the first use of [^{3}H]monofluoromethyl-Dopa ([^{3}H]MFMD), a new radiolabeled form of this enzyme-activated, irreversible inhibitor (Palfreyman et al., 1978; Jung et al., 1979; Sjoerdsma, 1981), for defining the subunit structure of human L-Dopa decarboxylase (DDC)[1] (L-aromatic amino acid decarboxylase, EC 4.1.1.28). DDC is a pyridoxal-containing enzyme that catalyzes the decarboxylation of certain aromatic L-amino acids (Lovenberg et al., 1962; Christenson et al., 1970, 1972). This protein has long been of interest to a diverse body of investigators for the following reasons: (a) in the central and peripheral nervous system of most species, it catalyzes the middle step in the synthesis of the monoamine neurotransmitters serotonin, dopamine, epinephrine, and norepinephrine; (b) some neurons, which do not contain the above monoamines, contain only DDC among the catecholamine-synthesizing steps and may synthesize important and yet to be characterized neurotransmitters directly from amino acid substrates such as tyrosine, phenylalanine, and tryptophan (Jaeger et al., 1983); (c) in insect species such as the drosophila, DDC is a temperature-regulated and possibly hormonally regulated enzymatic step in the synthesis of amine products that are necessary for proper formation of the cuticle (Kraminsky et al., 1980; Marsh & Wright 1980; Hirsh & Davidson, 1981); (d) DDC is an important phenotypic expression of widely dispersed endocrine cells that synthesize and secrete small polypeptide hormones (Pearse, 1969). In this latter regard, DDC is particularly important in humans because several types of cancer contain such DDC-rich endocrine cells [for review, see Pearse (1969) and Baylin & Mendelsohn (1980)]. The most common such tumor is the human small cell carcinoma of the lung, a virulent neoplasm that, at different stages of differentiation, manifests high DDC activity both in vivo (Berger et al., 1981) and especially in cell culture (Baylin et al., 1980; Goodwin & Baylin, 1982).

In order to learn more about the function of DDC in various cell types, including normal and neoplastic human endocrine cells, it is of interest to isolate and physicochemically characterize this human enzyme. To these ends, we now define the binding characteristics of a recently developed labeled preparation of the selective inhibitor of DDC, monofluoro-

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[1] Abbreviations: BSOCOES, bis[2-[[(succinimidooxy)carbonyl]-oxy]ethyl] sulfone; DDC, L-Dopa decarboxylase; Dopa, dihydroxyphenylalanine; MFMD, monofluoromethyl-Dopa; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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