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RESOLUTION OF INITIALLY EXCITED AND RELAXED STATES OF TRYPTOPHAN FLUORESCENCE BY DIFFERENTIAL-WAVELENGTH DECONVOLUTION OF TIME-RESOLVED FLUORESCENCE DECAYS

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We describe a new procedure for the analysis of time-resolved decays of fluorescence intensity. This procedure was used to resolve the emission spectra of the initially excited and solvent relaxed states of a tryptophan derivative in viscous solution. Specifically, we examined *N*-acetyl-L-tryptophanamide (AcTrpNH$_2$) in viscous and nonviscous solutions of propylene glycol. Time-resolved decays of fluorescence intensity were collected at wavelengths across the emission spectra. Instead of the usual procedure of deconvolving these data with the time profile of the exciting pulse, we deconvolved these data using the response observed on the short-wavelength side of the emission. If one assumes that this emission results only from the initially excited state (F), then the nonzero decay time calculated using deconvolution is that of the solvent relaxed state (R). For our specific case of AcTrpNH$_2$ the emission spectra of the F and R states overlap at most wavelengths longer than the short-wavelength side of the emission (310 nm). As a result, differential-wavelength deconvolution yields two lifetimes and amplitudes, one pair representing the relaxed state and the other the initially excited state. The latter appears as a zero-decay-time component whose amplitude can be readily quantified. The wavelength-dependent amplitude of this zero-lifetime component can be used to calculate the emission spectrum of the F state and, by difference, the emission spectrum of the relaxed state. For AcTrpNH$_2$ in propylene glycol at −20°C the emission maxima of the F and R states are near 320 and 350 nm, respectively, and the relative proportion of the emission from each state was near 50%. At lower temperatures the emission from the F state becomes dominant and at high temperatures the emission from the R state dominates. We note that this resolution of states is somewhat arbitrary because we assumed a two-state model and the absence of solvent relaxed emission at 310 nm. Nonetheless, differential-wavelength deconvolution simplifies and facilitates the analysis of time-resolved fluorescence data from samples which undergo excited state reactions. Moreover, this deconvolution procedure considerably simplifies the determination of the kinetic constants for reversible excited state reactions. The application of differential-wavelength deconvolution does not increase the time required for data acquisition. This differential analysis procedure should enhance the usefulness and precision of pulse fluorometric methods in studies of nanosecond time scale processes in proteins and membranes.

1. Introduction

Time-resolved fluorescence methods have been widely utilized in biochemical research. These measurements have been particularly valuable in studies of time-dependent processes such as excited state reactions and nanosecond spectral relaxation [1,2]. For example, Brand and co-workers reported on the excited state ionization of a naphthol derivative bound to human serum albumin [3] and on the relaxation of proteins and membranes around the excited states of bound fluorophores [4–6]. The wavelength-dependent time response of such systems may be used to calculate time-resolved emission spectra and the kinetic constants for the excited state reactions. These measurements can reveal the rates of spectral relaxation of solvent-sensitive fluorophores when bound to biological macromolecules, and hence the dynamic properties of proteins and membranes on the nanosecond time scale.

In the presence of an excited state process, the

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observed time-resolved intensities are complex functions, even following deconvolution with the lamp excitation profile. For example, assume that subsequent to excitation, an initially excited state F reacts reversibly, to form a relaxed state R. Assume further that the emission spectra of F and R do not overlap on the blue and red sides of the total emission. Then, the decays of both F and R are both double exponentials, each with the same decay times but differing preexponential factors. The two individual decay times are both functions of the forward and reverse reaction rates and the emissive rates of the F and R states. It is difficult to calculate the individual emission spectra from such data. Still greater complexity is encountered for the case of solvent relaxation. The time-dependent spectral shifts may occur via a series of smaller steps [7] or continuously [8]. In either event the decay is nonexponential and resolution of individual spectra is difficult.

In the following sections we describe a new method of analyzing time-resolved data to identify the emission spectrum of the initially excited and solvent relaxed states, and to measure directly the intrinsic lifetime of the relaxed state, assuming this state could be excited directly. The usual procedure in pulse fluorometry is to use the time profile of the excitation pulse for deconvolution with the fluorescence emission. Instead of the excitation pulse we used the observed response of the F state, or its equivalent, the observed response on the blue side of the emission. The emission at longer wavelengths was deconvolved using the time response of the short-wavelength emission. This procedure results in considerable simplification of the calculated impulse response of the R state. For an irreversible or reversible excited state reaction, the double-exponential decay of the R state is reduced to a single-exponential decay, with a lifetime equal to the intrinsic lifetime of the R state [9]. Frequently, the emissions of the F and R states overlap, further complicating the double-exponential decay of each species. However, use of the differential-wavelength deconvolution reduces the emission of the F state to an easily identified and quantified zero-decay-time component irrespective of the complexity of the time-resolved response of the F state. This simplification occurs because the initially excited F state is used as the 'excitation function' in the deconvolution. Thus, any spectral contribution from this state at longer wavelengths has the same time response as the excitation function, and hence a zero decay time. This simplified data set allows the emission spectra of the F and R states to be easily calculated. In addition, the double-exponential decay of the R state becomes a single-exponential decay and the decay time is characteristic of only the R state.

Eventually, it is hoped that this procedure of differential-wavelength deconvolution will prove valuable in the analysis of spectral relaxation of tryptophan fluorescence from proteins. To model the proteins we chose *N*-acetyl-L-tryptophanamide ($AcTrpNH_2$). In fluid solvents the decay of fluorescence from $AcTrpNH_2$ is well approximated by a single lifetime [10,11]. In viscous solutions the apparent lifetimes of $AcTrpNH_2$ are dependent upon emission wavelength, and this dependence was demonstrated to result from solvent reorientation around the excited state [12,13]. We used differential-wavelength deconvolution to calculate the emission spectra of the initially excited and the solvent relaxed states of $AcTrpNH_2$ in propylene glycol. At −20°C approximately equal intensities were found for each state. In fluid and vitrified solvent the emission resulted predominantly from the relaxed and initially excited states, respectively. The ability of our procedure to reveal even small spectral components was illustrated by the presence of a small percentage (10%) of solvent relaxed emission at −60°C. Hence, we expect differential-wavelength deconvolution to enhance the usefulness of pulse fluorometric methods in the analysis of a variety of excited state processes in proteins and membranes, especially those instances where the detection of minor spectral components is required.

2. Theory

Consider the simple model shown in fig. 1. We assume that the initially excited state F decays by both emission with a rate constant Γ and by relaxation to the solvent relaxed state R with a rate constant *k*. For simplicity, we did not ex-

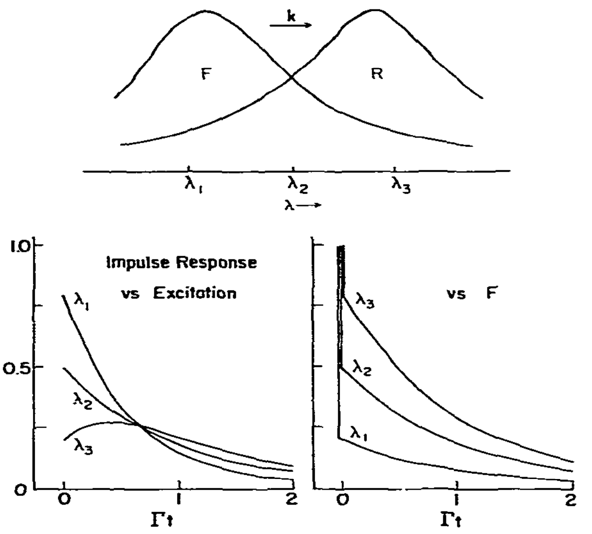

Fig. 1. Pictorial representation of time-resolved data for irreversible excited state reaction and differential-wavelength deconvolution of these data.

plicitly include the nonradiative decay rates and we further assumed that the relaxation is irreversible. This model is described by the following kinetic equations:

$$\frac{dF(t)}{dt} = -(\Gamma + k)F(t) + E(t) \tag{1}$$

$$\frac{dR(t)}{dt} = -\Gamma R(t) + kF(t) \tag{2}$$

where $F(t)$ and $R(t)$ are the excited state populations and $E(t)$ the time profile of the exciting pulse. The fluorescence intensities $I_F(t)$ and $I_R(t)$ may be obtained from the number of molecules in the F and R states using $I_F(t) = \Gamma F(t)$ and $I_R(t) = \Gamma R(t)$. The solutions for the time-resolved decays of the intensity from F and R are:

$$I_F(t) = \Gamma e^{-t/\tau_F} \tag{3}$$

$$I_R(t) = \Gamma[e^{-t/\tau_R} - e^{-t/\tau_F}]. \tag{4}$$

These expressions are normalized to a total quantum yield of unity. The relative quantum yields of the F and R states, given by the time-integrated intensities, are $\Gamma/(\Gamma + k)$ and $k/(\Gamma + k)$, respectively. The lifetimes are $\tau_F = (\Gamma + k)^{-1}$ and $\tau_R = \Gamma^{-1}$.

One may demonstrate formally that when a relaxed state is populated from an initially excited state, then the population of the relaxed state is given by the convolution of its intrinsic decay $R^0(t)$ with the population of the initially excited state:

$$R(t) = k\int_0^t F(t - t')R^0(t')\,dt. \tag{5}$$

This result is obtained irrespective of the complexity of time-dependent population of the initially excited state. Instead of a formal proof we chose to demonstrate that the double-exponential decay of $I_R(t)$ (eq. 4) follows from eq. 5. The intrinsic decay of R, if this species could be excited directly, is given by

$$I_R^0 = \Gamma e^{-t/\tau_R} \tag{6}$$

Substitution of eqs. 3 and 6 into eq. 5 yields eq. 4. This result demonstrates that the double-exponential decay of $I_R(t)$ is a result of the convolution of the population of the R state with the population of the F state.

The implication of this concept (eq. 5) is as follows. Assume one obtains the time-resolved decays of F and R. Then deconvolution of $I_R(t)$ using $I_F(t)$ as the excitation function yields the intrinsic decay of the R state. This result is quite general and can be applied in other instances where a given species is formed from a previous species, irrespective of the number of steps. Examples which come to mind are kinetic pathways in multiple-step enzyme-catalyzed reactions, excimer and exciplex formation and energy transfer among the fluorophores. Differential-wavelength deconvolution results in simplification of the measured parameters and hence an improved capability of analyzing such complex processes. In particular, the derived impulse response function reveals the intrinsic lifetime of the relaxed state.

In our model we assumed that the reaction was irreversible. As a result the decay of the F state was a single exponential and differential-wavelength deconvolution revealed the lifetime of the R state. We note that this result is also obtained for a reversible reaction, as described elsewhere in more detail [9]. For reversible excited state reactions the decay of both the F and R states is doubly exponential at all wavelengths and the decay rates are constant across the emission spectrum [14]. In contrast to the irreversible reaction described above, these decay rates are not characteristic of either state, i.e., both wavelength-independent decay rates are dependent upon the forward and reverse reaction rates and the emissive rates of the F and R states. As a result it is difficult to interpret the measured parameters in terms of the spectra and kinetic constants of the system. Importantly, differential-wavelength deconvolution of the R state vs. the F state yields uniquely the intrinsic lifetime of the R state, irrespective of the reversibility of the reactions or the double-exponential decay of F. Hence, this procedure also results in simplification of the analysis of reversible excited state reactions.

In the current study of $AcTrpNH_2$ the emission of the F state overlaps substantially with that of the R state. As the emission wavelength is increased from 310 nm the fractional contribution of the F state decreases monotonically. This emission, since it is directly excited, appears as a zero-decay-time component when the time-resolved decays are analyzed by the differential-wavelength deconvolution. The fractional intensity of this component decreases as the emission wavelength is increased and the emission from the R state becomes more significant. The zero-decay-time component is easily quantified and the magnitude of this component in the time-resolved decay indicates the fractional contribution of the F state at any given wavelength. Specifically, differential-wavelength deconvolution reveals the two lifetimes and amplitudes in:

$$I(t)=\alpha_R e^{-t/\tau_R}+\alpha_0 e^{-t/\tau_0} \tag{7}$$

where α_R and τ_R are the amplitude and lifetime of the R state, respectively, and α_0 and τ_0 those of the initially excited state. In practice, the zero-decay-time component appears as a short-lived decay time of about 0.1 ns. The accuracies of α_0 and τ_0 individually are poor, but the product is quite reproducible. The emission spectrum of the initially excited state can be calculated from

$$I_F(\lambda)=\frac{\alpha_0\tau_0}{\alpha_0\tau_0+\alpha_R\tau_R}I(\lambda) \tag{8}$$

where $I(\lambda)$ is the steady-state emission spectrum. The spectrum of the relaxed state is calculated from:

$$I_R(\lambda)=\frac{\alpha_R\tau_R}{\alpha_0\tau_0+\alpha_R\tau_R}I(\lambda) \tag{9}$$

Considerably more complex expressions are required to calculate the individual spectra in the presence of either a reversible or an irreversible excited state reaction [9].

These concepts are illustrated in fig. 1. For the irreversible reaction the time-resolved decay of the F state, following extremely short pulsed excitation, is a single exponential with a rate constant $\Gamma+k$. The intensity of the R state starts at zero, builds up to a maximum, and then decays exponentially at longer times with a rate constant Γ. In the right panel of fig. 1 we illustrate the appearance of these data when deconvolved vs. the F state. More precisely, these plots represent the derived impulse response functions. Of course, when deconvolved vs. the F state the emission from the F state appears as a zero-decay-time component. This component is illustrated by the rapid vertical decay. The fractional intensity of this zero-lifetime component represents the fractional contribution of the initially excited state at the emission wavelength being observed. As the observation wavelength is increased the magnitude of this component decreases. Finally, on the red side of the spectrum the emission from the solvent relaxed state dominates and deconvolved response is a single exponential with a rate constant Γ.

3. Materials and methods

Time-resolved decays of fluorescence intensity were obtained using a single-photon pulse fluorometer from Photochemical Research Associates. Deconvolution was performed using the method of least squares [15,16]. For each measurement the time-resolved intensities were collected for the exciting pulse, for the blue edge of the emission (310 nm) and for the longer wavelength of interest. The longer wavelength decay was deconvolved using both the light pulse and the 310 nm data. Steady-state spectra were obtained on a fluorometer from SLM Instruments, Inc. For all experiments the excitation wavelength was 280 nm, and the excitation and emission band passes were 8 nm. Polarizers were not used in either the excitation or emission light paths. The absorbance of the $AcTrpNH_2$ solution was 0.3. Relative to the fluorescence from $AcTrpNH_2$, the background fluorescence from the propylene glycol solvent was less than 1% at all measured wavelengths.

4. Results

Emission spectra of $AcTrpNH_2$ in propylene glycol are shown in fig. 2. At high temperature the emission maximum is 350 nm. Cooling this solution to −60°C results in a blue shift in the emission spectrum, and an emission maximum of 327 nm. At an intermediate temperature (−20°C) an intermediate spectrum is observed with an emission maximum of 341 nm. We attribute these temperature-dependent spectral shifts to solvent relaxation around the excited state of $AcTrpNH_2$ [12,13]. At low temperature, relaxation occurs more slowly than emission ($k \ll \Gamma$) and emission results primarily from the F state. At high temperature, relaxation occurs more rapidly than emission, and the emission is dominated by that of the R state. At intermediate temperature, emission occurs from both states with the relative proportion of the F and R states being $\Gamma/(\Gamma+k)$ and $k/(\Gamma+k)$, respectively. We stress that the terms F and R states are being used to simplify discussion. In reality, it is likely that emission occurs from a number of partially relaxed states, which are not resolvable with the data presently available.

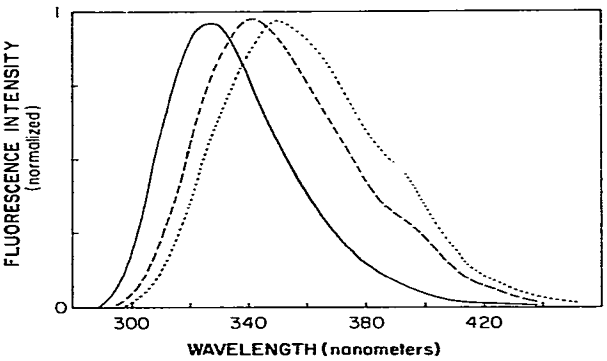

Fig. 2. Steady-state emission of *N*-acetyl-L-tryptophanamide in propylene glycol. (———) −60°C, (— — —) −20°C, (------) +40°C.

In table 1 we summarize our time-resolved measurements for $AcTrpNH_2$ when these data are deconvolved vs. the excitation pulse. The data were analyzed in terms of a double-exponential decay, but we note that this analysis provides only an approximate representation of what is likely to be a more complex decay law. At −20°C two decay times were needed to fit the data. The fractional amplitude of the longer decay-time term increased with emission wavelength. However, we note that in the presence of an excited state reaction the $\alpha\tau$ products do not represent the relative intensities of the individual states. At +40 and −60°C the time-resolved decays of intensity were well represented by a single decay time irrespective of emission wavelength.

It is interesting to note that the term with a negative preexponential factor was not observed at −20°C, even though the wavelength-dependent lifetimes were proven to be a result of solvent relaxation [12,13]. Furthermore, one could conceivably analyze such data in terms of ground state heterogeneity rather than solvent relaxation. In our opinion, it is difficult to distinguish relaxation from heterogeneity using the time-resolved decays of tryptophan fluorescence [17]. This difficulty is probably a result of the large degree of spectral overlap of the unrelaxed and relaxed emission spectra and the short lifetime of tryptophan fluorescence.

Examination of fig. 2 reveals that at −60°C the emission at 310 nm is dominated by emission from the unrelaxed state. The intensity at 310 nm is 7-fold larger at −60°C than at +40°C. We used the time response at 310 nm for deconvolution of the time-resolved decays measured at longer wavelength and these data are also summarized in table 1. At −20°C a double-exponential decay is still required to fit these data using differential-

Table 1

Time-resolved decays and differential-wavelength deconvolution of *N*-acetyl-L-tryptophanamide in propylene glycol

Temperature (°C)	Wavelength (nm)	Deconvolution versus					
		Excitation pulse			Response at 310 nm		
		τ_1 (ns)	τ_2 (ns)	$\alpha_2\tau_2$ (%)	τ_0 (ns)	τ_R (ns)	$\alpha_0\tau_0$ (%)
−20	310	1.4	5.5	58	–	–	–
	20	1.8	6.4	65	0.03	4.9	84
	30	1.4	6.5	77	0.06	5.1	73
	40	2.3	7.1	80	0.08	5.4	61
	50	–	6.9	94	0.05	5.6	53
	60	3.9	7.6	87	0.18	5.8	47
	70	5.0	7.9	83	0.23	6.0	44
	80	–	7.7	98	0.31	6.1	41
	90	–	7.9	100	0.41	6.2	36
+40	310	0.2	3.8	89	–	–	–
	30	1.0	3.8	91	0.21	–	100
	50	1.0	3.7	96	0.28	–	100
	70	0.5	3.8	97	0.28	–	100
	90	–	3.8	99	0.30	–	100
	410	–	–	99	0.31	–	100
−60	310	0.01	4.8	94	–	–	–
	30	0.01	5.1	92	0.01	5.5	96
	50	0.01	5.4	93	0.02	6.0	92
	70	0.002	5.6	93	0.02	5.5	87
	90	0.08	4.8	95	0.14	6.0	87

wavelength deconvolution, but now one of the decay times is near zero. The fractional intensity of the zero-decay-time component decreases with increasing observation wavelength. We attribute this zero-decay-time component to emission from the initially excited state and we attribute the second longer lived component to the emission from the solvent relaxed state. We used these data to calculate the emission spectra of the F and R species, as described by eqs. 8 and 9. These spectra (fig. 3) display an emission maximum at 330 and 350 nm for the F and R states, respectively. The calculated spectra are similar to the steady-state spectra observed at −60 and +40°C. More detailed data collection and analysis is required to judge whether this agreement is significant or circumstantial. An additional resolution of the emission from the F and R states is shown in the lower panel of fig. 3. At −60°C the time-resolved data, when deconvolved vs. the blue edge emission, show about 90% of the emission as a near zero-decay-time component, plus a small proportion with a 5–6 ns lifetime (table 1). The emission spectrum of this longer lived component is seen to have an emission near 350 nm, as is expected for the solvent relaxed state.

At 40°C the time-resolved decays, when deconvolved vs. the blue edge, are also dominated by a zero-decay-time component. This is expected, since at 40°C solvent relaxation is expected to be rapid and then the emission is dominated by the relaxed state. Hence, we see that under conditions where the emission is predominantly due to a single species the apparent decay time resulting from differential-wavelength deconvolution is zero. It appears that this deconvolution procedure will be valuable in analyzing time-resolved data for the presence of small components formed subsequent to excitation. Such small components are likely for tryptophan fluorescence from proteins [17] and possibly from ionized tyrosine residues [18].

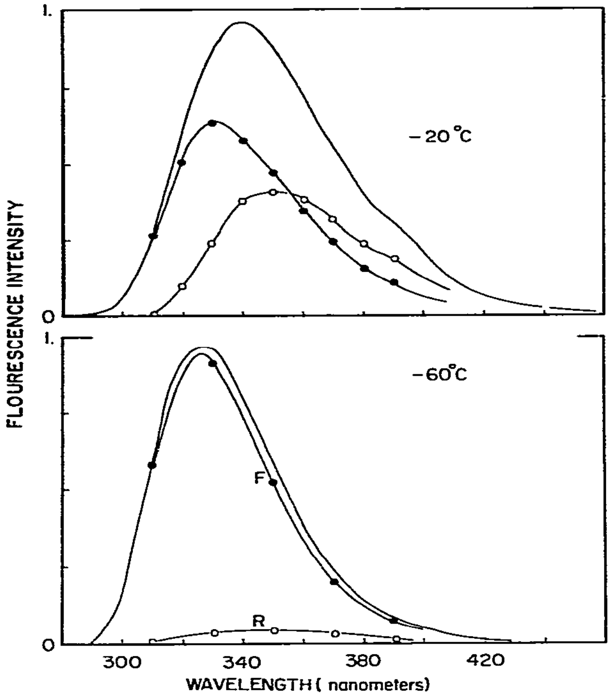

Fig. 3. Resolution of the emission spectra of the initially excited and solvent relaxed states of AcTrpNH$_2$ in propylene glycol by differential-wavelength deconvolution. (———) Steady state: differential-wavelength deconvolution of (●) F state and (○) R state.

5. Discussion

Other researchers have used pulse fluorometry methods to resolve the individual emission spectra of tryptophan from proteins which contain two tryptophan residues such as horse liver alcohol dehydrogenase and yeast 3-phosphoglycerate kinase [19,20]. In these studies the authors used expressions of the form:

$$I_i(\lambda)=\frac{\alpha_i\tau_i}{\alpha_1\alpha_2+\alpha_2\tau_2}I(\lambda) \qquad (10)$$

where the subscripts refer to the individual tryptophan residues. α_1 and α_2 are dependent upon emission wavelength but τ_1 and τ_2 are generally restricted to be independent of wavelength. This expression is similar to our eqs. 8 and 9, but we note that these are distinct situations. In the studies of the two tryptophan proteins the origin of the wavelength-dependent time response was the ground state heterogeneity resulting from the presence of two tryptophan residues. For ground state heterogeneity eq. 10 is valid. In our case the origin of the wavelength-dependent time response was solvent relaxation on the nanosecond time-scale. Then the wavelength-dependent data, when deconvolved with the exciting pulse, cannot be used with eq. 10 to calculate the individual spectra. More complex procedures are necessary [9] and such procedures may become available in the near future [21]. Presently, such procedures are not generally available. A significant advantage of differential-wavelength deconvolution is the simplification of the data to allow calculation of the individual spectra in systems which display time-dependent spectral shifts. We expect differential-wavelength deconvolution to enhance the usefulness of pulse fluorometric data in the analysis of spectral relaxation of fluorophores bound to proteins and membranes.

Differential-wavelength deconvolution is not limited to studies of solvent relaxation. In this report we used a tryptophan derivative to illustrate the ease with which individual spectra may be calculated even for the difficult case of tryptophan fluorescence. In another report [9] we describe in more detail the general aspects of differential-wavelength deconvolution and demonstrate its application to the analysis of reversible and irreversible excited state reactions. Such processes are frequently encountered in fluorescence studies of biological macromolecules. The application of differential-wavelength deconvolution does not require additional data acquisition, only further analysis of data which have already been collected. This fact, coupled with the simplification of the calculated parameters, should result in widespread application of this new procedure.

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