Study of Triplet–Singlet Energy Transfer in an Enzyme–Dye Complex Using Optical Detection of Magnetic Resonance†

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ABSTRACT: We have made optical detection of magnetic resonance (ODMR) measurements on the enzyme α-chymotrypsin, as well as on its complex with the dye, proflavin. Evidence that triplet–singlet energy transfer occurs in the complex is provided by the observation of characteristic tryptophan ODMR signals while monitoring the delayed fluorescence of the dye. The luminescence decay kinetics of the complex indicates that nontrivial triplet–singlet transfer originates from several (at least three) tryptophan residues of the enzyme. ODMR sensitivity can be enhanced by coupling the sublevels of a weakly radiative triplet state to a fluorescent dye which satisfies Förster's (Förster, T. (1948), *Ann. Phys. (Leipzig) 2,* 55; (1965), in Modern Quantum Chemistry, Istanbul Lectures, Part III, Sinanoglu, O., Ed., New York, N.Y., Academic Press, p 93) conditions for energy transfer.

Triplet–singlet energy transfer has been demonstrated to occur in DNA–acridine dye complexes (Isenberg et al., 1964) as well as in the 1:1 complex (Bernhard et al., 1966) of the enzyme α-chymotrypsin with the acridine dye, proflavin (Galley and Stryer, 1969). Aside from the trivial process of absorption of phosphorescence quanta by the acceptor followed by reemission as fluorescence and phosphorescence, energy transfer via dipole–dipole coupling of donor and acceptor transition dipoles can lead to triplet–singlet energy transfer over distances the order of 40 Å (Förster, 1948, 1965; Ermolaev and Sveshnikova, 1963). Careful experimental work (Kellogg, 1967) has established the essential validity of Förster's dipole–dipole model in random solid solution model systems.

According to the theory, the rate constant for energy transfer between an excited state donor molecule and an acceptor molecule may be expressed as

$$k^{tr} = (8.71 \times 10^{23})kQr^{-6}K^2Jn^{-4}\ s^{-1} \qquad (1)$$

where k is the overall decay constant of the donor excited state in the absence of energy transfer, while Q is its radiative quantum efficiency. kQ may be identified as the radiative rate constant, k^r. r is the distance between the centers of the donor and acceptor molecules, n is the refractive index, and K^2 is an orientation factor given by

$$K^2 = (\cos\gamma - 3\cos\alpha\cos\beta)^2 \qquad (2)$$

where γ is the angle between donor and acceptor transition moments, while α and β are the angles between the line joining the molecular centers and the transition moments of the donor and acceptor molecules, respectively. J is a measure of the overlap of the emission spectrum of the donor, $I(\lambda)$, and the absorption spectrum of the acceptor, $\epsilon(\lambda)$, and is given by

$$J = \frac{\int I(\lambda)\epsilon(\lambda)\lambda^4 d\lambda}{\int I(\lambda)d\lambda} \qquad (3)$$

There is no limitation on the multiplicity of the donor, and it is clear from eq 1 and the subsequent definition of its terms that, given a comparable spectral overlap factor, J, and orientation factor, K^2, the energy transfer efficiency, $k^{tr}/(k^{tr} + k)$ is independent of k for excited states of comparable Q. In other terms, the effective range of triplet–singlet transfer by the Förster mechanism is basically no different than that of singlet–singlet transfer—typically about 40–50 Å.

Evidence presented for triplet–singlet energy transfer in the α-chymotrypsin–proflavin complex was the observation that at 77 K (a) delayed dye fluorescence accompanies the optical excitation of the aromatic chromophores of the enzyme (principally tryptophan), but is not present when the dye itself is optically pumped at longer wavelengths, and (b) the tryptophan phosphorescence/fluorescence ratio is reduced below that found in the enzyme itself (Galley and Stryer, 1969).

Although measurement of triplet–singlet transfer rate constants leads to the same type of information regarding the spacial relationship of the chromophores as does the measurement of singlet–singlet rate constants, the longer time scale for triplet–singlet transfer measurements makes them simpler experimentally, and potentially more accurate. A special feature exhibited by an excited triplet state when acting as an energy donor is the spin multiplicity which leads to three distinct sublevels, T_i, in zero field, where $i = x, y,$ or z. In these sublevels, the spin angular momentum is polarized in the planes of the zero-field splitting principal axis system of the molecule. These sublevels have distinct and distinguishable properties including different energies, E_i, due to magnetic dipole–dipole coupling, as well as different intersystem crossing rates, P_i, decay rate constants, k_i, and radiative rate constants, k_i^r (van der Waals and de Groot, 1967), because of spin sublevel-dependent spin–orbit coupling routes. It is these distinguishable properties which make possible optical detection of magnetic resonance (ODMR[1]) experiments in which the phospho-

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[1] Abbreviations used are: CRT, cathode ray tube; ODMR, optical detection of magnetic resonance; EEDOR, electron–electron double resonance.

rescence intensity may be modulated as a result of magnetic resonance transitions induced between the triplet sublevels (Sharnoff, 1967; Kwiram, 1967). The ODMR experiments usually require low temperatures in the liquid helium range to suppress spin–lattice relaxation processes and thereby allow the establishment of significant population differences among the sublevels by selective populating and decay routes. At temperatures as high as 77 K which have been common for measurements of EPR and phosphorescence of triplet states, spin–lattice relaxation rate constants are much larger than the k_i so that only average triplet rate constants are measurable. Except for transients present briefly upon commencement of optical pumping (Levanon and Weissman, 1971), spin–lattice relaxation maintains the sublevel populations in Boltzmann equilibrium, i.e., nearly equal at 77 K. Generally, the triplet–singlet transfer rate constant for a donor–acceptor pair with a specific spacial relationship will be different for each triplet sublevel because the transfer rate constant, k_i^{tr}, is proportional to k_i^r as well as to the orientation factor, K_i^2. The condition $k_i^r \neq k_j^r$ is a general requirement for ODMR signals to be observed when pumping the $T_i \leftrightarrow T_j$ EPR transition with microwaves, whereas $K_i^2 \neq K_j^2$ if the dipole moments of the transitions $S_0 \leftarrow T_i$ and $S_0 \leftarrow T_j$ are differently polarized.[2] It is clear (provided none of the k_i^{tr} approaches the spin–lattice relaxation rate constants) that at 77 K only the average triplet–singlet transfer rate constant, $\bar{k}^{tr} = (k_x^{tr} + k_y^{tr} + k_z^{tr})/3$, will be observed. Consequently, at temperatures low enough to quench spin–lattice relaxation, more information is potentially available from measurements of triplet–singlet than from singlet–singlet transfer since three donor excited states with generally differently polarized transition moments may be located on the same molecular framework. Other features of interest when spin–lattice relaxation is quenched are: (a) with the k_i^{tr} unequal for the sublevels, it is possible to affect the acceptor fluorescence intensity by means of microwave-induced transitions which change the sublevel populations of the donor triplets; i.e., *the detection of magnetic resonance of a triplet donor by monitoring the delayed fluorescence of an energy acceptor is possible;* (b) ODMR signals which are weak because of a small k_i^r may be enhanced by coupling of the transition dipole of the sublevel with that of a radiative singlet acceptor molecule through Förster transfer; and (c) ODMR signals which are weak because of a relatively small difference of populations among the sublevels in the steady state may become stronger due to the generation of large spin alignments by sublevel-selective energy transfer. Thus, aside from spacial information available from studying the kinetics of triplet–singlet transfer, *the coupling of fluorescent dye molecules to triplet states through dipole–dipole interaction can greatly enhance the sensitivity of ODMR detection. In addition, by monitoring the delayed fluorescence, the identity of a triplet donor molecule can be established unequivocally among a number of possibilities from measurement of the microwave frequencies which elicit optical responses.*

We report here on our initial measurements of the luminescence and ODMR of the complex α-chymotrypsin–proflavin in which we prove that triplet–singlet energy transfer from tryptophan to proflavin occurs by detecting tryptophan zero-field magnetic resonance transitions by monitoring the proflavin delayed fluorescence. Also, we demonstrate feature b above by observing the $D + E$ signal of tryptophan with delayed fluorescence detection. We can not observe this signal by monitoring the tryptophan phosphorescence since k_z^r and k_y^r both are small (Zuclich et al., 1973).

Materials and Methods

Proflavin sulfate and three times recrystallized α-chymotrypsin were obtained from Schwarz/Mann, Inc. Proflavin sulfate was recrystallized from water in the dark, and the α-chymotrypsin and proflavin sulfate were dissolved in a 1:1 (v/v) mixture of ethylene glycol and 2×10^{-2} M potassium phosphate buffer, pH 6.8. The resulting solution was adjusted back to pH 6.8 by adding tiny amounts of potassium phosphates. A 5×10^{-4} M solution of α-chymotrypsin was made in the same solvent. The α-chymotrypsin–proflavin solution was equilibrated in the dark overnight at 4 °C before it was cooled further to form a rigid glass. Samples were run in a 1-mm quartz tube.

The sample, inserted into a microwave slow-wave helix terminating a coaxial transmission line, was suspended in a Dewar which could be maintained at 77 K, 4.2 K, or temperatures below 4.2 K by pumping on the liquid helium. The samples were excited with a 100-W high-pressure Hg arc filtered with a quartz prism monochromator. The luminescence was monitored at right angles with a McPherson Model 2051 grating monochromator and detected by a cooled EMI Model 9558QA photomultiplier. The microwave source was a Hewlett-Packard 8690B sweep oscillator fitted with the appropriate plug-in for the desired frequency range.

For luminescence decay measurements or microwave-induced delayed luminescence measurements (Schmidt et al., 1971), a digital multipulse time base was used to trigger a Nicolet, Inc., Model 1072 signal averager, a shutter in the optical excitation path, one in the luminescence path, and a rapid (10 ms) microwave frequency sweep. The response time of the shutters was ca. 1 ms. In the deconvolution of the luminescence decays, about 900 data points were included in each decay which was carried out over a 35–40 s time interval and which included a dynamic range of about three orders of magnitude in light intensity. Deconvolution of the decays into the minimum number of exponential components required to fit the experimental data to within the noise was accomplished with the aid of a computer graphics program. A plot of the logarithm of the intensity vs. t was initially displayed on a cathode ray tube (CRT[1]) terminal. The tail of the decay was fit by superimposing a straight line directly on the CRT display; the computer then subtracted this line from the initial data and displayed a plot of the difference on the CRT. The process was repeated until no significant (outside of noise) intensity remained in the difference plot. Each step resulted in the elimination of one exponential component from the data. Finally, the computer prepared a theoretical decay curve from the components obtained from the deconvolution, subtracted this from the experimental data, and displayed the difference vs. t on the CRT. If the difference plot was observed to be zero within the noise, the analysis was considered satisfactory. If not, the deconvolution was repeated with changes suggested by the difference plot.

For ODMR slow-passage measurements, a rotating sector was substituted for the shutters to eliminate scattered

[2] If emission from a sublevel, T_j, is only vibronically allowed to the ground state, its spectral overlap integral, J_j, also may be significantly different from that of a sublevel whose O–O emission is allowed.

light and immediate fluorescence, and the time base was used to synchronize the signal averager sweep and the (slow) microwave frequency sweep. For observation of the enzyme phosphorescence, the McPherson monochromator is set at 413 nm, while for delayed fluorescence of the dye it is set to the fluorescence peak at 485 nm.

EEDOR (electron-electron double resonance) measurements were performed as described previously (Zuclich et al., 1974a; Kuan et al., 1970).

Microwave-saturated phosphorescence decay measurements were carried out on α-chymotrypsin at 1.2 K, monitoring the phosphorescence at 413 nm. Details of the method, and analysis of the decays to yield the sublevel decay rate constants, and the spin-lattice relaxation rate constants have been reported previously (Co et al., 1974; Zuclich et al., 1974b).

Results

α-Chymotrypsin. The phosphorescence of α-chymotrypsin when excited at 290 nm was similar at 1.2, 4.2, and 77 K and appeared to be due entirely to tryptophan. The sharp O-O band peaked at 413 nm; the phosphorescence decay accumulated at this wavelength was a single exponential over more than two decades of intensity with a decay constant of 0.170 s^{-1} at 77 K and 0.162 s^{-1} at 4.2 K. At 1.2 K, the decay became complex, indicating that uncoupling of the sublevels due to a reduction in spin-lattice relaxation rate constants has occurred at the lower temperature. Slow-passage ODMR signals during continuous optical pumping for the $T_x \leftrightarrow T_z$ $(D - E)$ and $T_x \leftrightarrow T_y$ $(2E)$ transitions are shown in Figures 1a and 1b, respectively. No signal can be observed for the $T_y \leftrightarrow T_z$ $(D + E)$ transition either by slow passage during continuous optical pumping, or by microwave fast passage during triplet-state decay (Schmidt et al., 1971). A strong $D + E$ signal was observed using the EEDOR method, however. Figure 1c shows the EEDOR-induced slow passage $D + E$ signal while simultaneously and continuously frequency modulating a second saturating microwave frequency through the 2E signal region (Figure 1b). It is interesting that the EEDOR signal can not be observed by saturating the $D - E$ signal region. This is because the average of the populations of T_z and T_x in the photostationary state is very nearly equal to that of T_y (Zuclich et al., 1974b).

The observation that the phosphorescence of the enzyme decays as a single exponential at 4.2 and 77 K to within our ability to discriminate suggests that all phosphorescent tryptophan chromophores have the same average decay constant, $\bar{p} = (k_x + k_y + k_z)/3$, within experimental error. This is consistent with previous work (Longworth, 1971; Zuclich et al., 1974b; Maki and Zuclich, 1975), indicating that the tryptophan triplet lifetime is relatively insensitive to the environment. In order to determine whether the low-temperature (1.2 K) luminescence could be fit to a single set of sublevel decay constants (k_i) and spin-lattice relaxation rate constants (W_{ij}), microwave saturated phosphorescence decay measurements (Co et al., 1974; Zuclich et al., 1974b) were carried out on the enzyme at 1.2 K. Although we cannot tell for certain how much heterogeneity in these kinetic parameters exists among the emitting tryptophan sites of α-chymotrypsin, the microwave-saturated decays could be fit to a single set of k's and W's. The results for k_x, k_y, k_z, W_{xy}, W_{yx}, W_{xz}, W_{zx}, W_{yz} and W_{zy} are 0.316, 0.132, 0.034, 0.000, 0.000, 0.067, 0.063, 0.019, and 0.016 s^{-1}, respectively. The sublevel decay constants and spin-lattice relaxation pattern are similar to those found previously for the phosphorescent tryptophans of hen lysozyme (Zuclich et al., 1974b). Since we cannot tell to what extent these kinetic parameters are characteristic of *each* phosphorescent tryptophan of α-chymotrypsin, they should be considered as an average property of the enzyme.

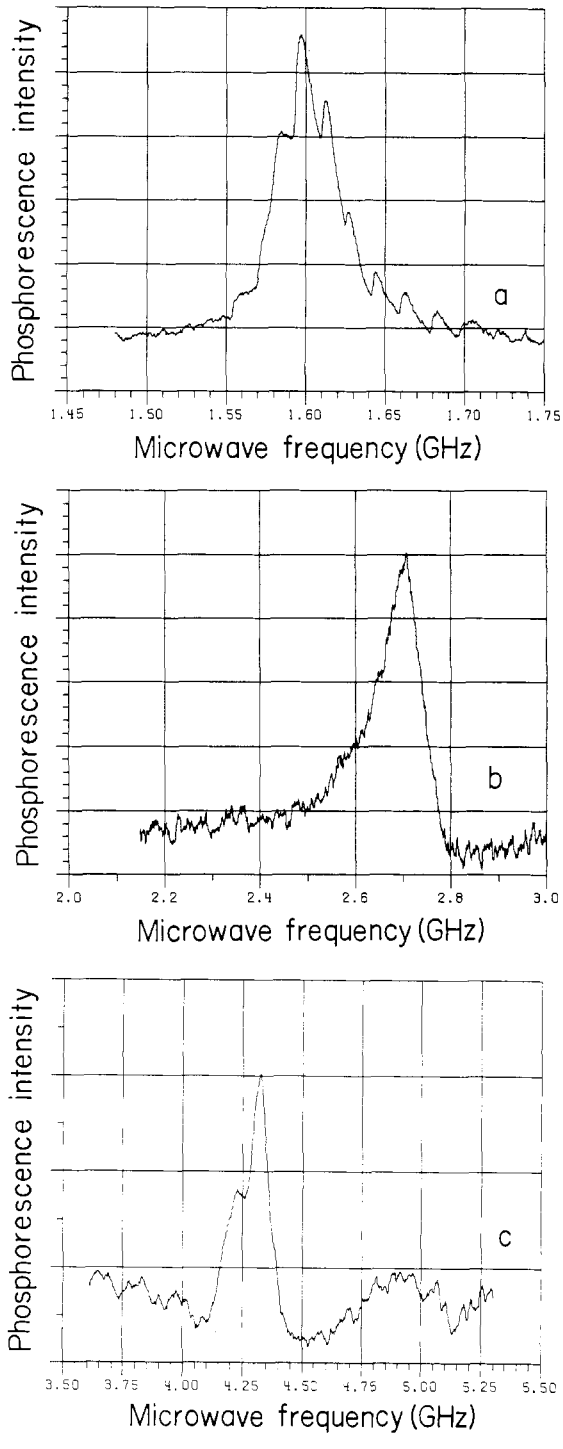

FIGURE 1: Slow-passage ODMR signals observed in α-chymotrypsin while monitoring the tryptophan phosphorescence peak at 413 nm. Concentration is 5×10^{-4} M in a pH 6.8 phosphate buffer containing an equal volume of ethylene glycol; $T = 1.14$ K. Microwave scan time is 90 s: (a) $(D - E)$ signal; (b) $(2E)$ signal; (c) $(D + E)$ signal observed by EEDOR while simultaneously saturating the $2E$ signal.

α-Chymotrypsin-Proflavin. The delayed luminescence spectra observed at 77 K for the enzyme-dye complex are shown in Figure 2. The emission spectra at 4.2 and 1.2 K

Table I: Decay Analysis of Phosphorescence and Delayed Fluorescence of the α-Chymotrypsin-Proflavin Complex.[a]

Phosphorescence (Monitor 413 nm)			Fluorescence (Monitor 485 nm)		
Preexponential Factor (%)	Decay Constant (s^{-1})	Transfer Constant (s^{-1})[b]	Preexponential Factor (%)	Decay Constant (s^{-1})	Transfer Constant (s^{-1})[b]
40.3 (0.9)	0.167 (0.001)	−0.003	13.3 (1.1)	0.168 (0.004)	−0.002
34.3 (1.1)	0.337 (0.022)	0.167	18.2 (1.3)	0.383 (0.018)	0.213
25.4 (1.5)	1.39 (0.20)	1.22	32.4 (3.8)	1.28 (0.13)	1.11
			36.1 (4.2)	3.86 (0.47)	3.69

[a] T = 77 K. Excitation at 290 nm. Concentrations of dye and enzyme each are 5×10^{-4} M in a pH 6.8 phosphate buffer. All decays are followed for 40 s. Data are the average of five independent measurements with exciting light intensities varying over a factor of 15. Standard deviations are given in parentheses. [b] Calculated from $k^{tr} = k^{tot} - k$, where k^{tot} is the entry in the previous column, and k (= 0.170 s^{-1}) is the observed single decay constant of α-chymotrypsin at 77 K.

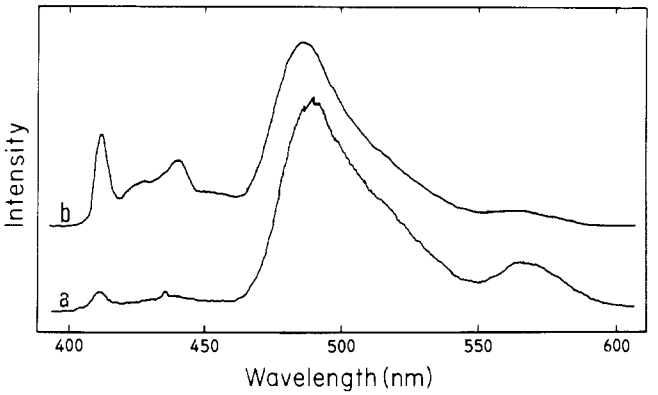

FIGURE 2: Delayed luminescence spectra of α-chymotrypsin-proflavin complex at 77 K; excitation at 290 nm. Enzyme concentration is 5×10^{-4} M: (a) proflavin concentration is 5×10^{-4} M; (b) proflavin concentration is 1×10^{-4} M. Both spectra are taken in a pH 6.8 phosphate buffer, mixed with an equal volume of ethylene glycol.

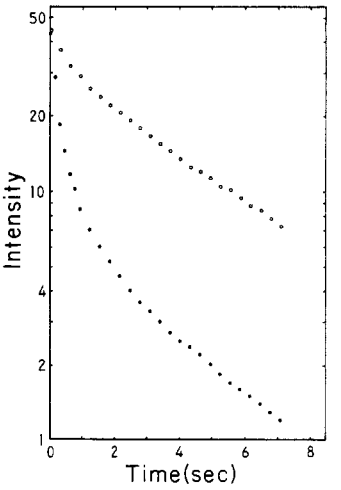

FIGURE 3: Luminescence decay of α-chymotrypsin-proflavin complex monitoring at 413 nm (○) and at 485 nm (●). Conditions are as in Figure 2a.

are the same. The spectra are similar to those observed previously (Galley and Stryer, 1969). The emission between 400 nm and 460 nm is tryptophan phosphorescence, while the strong band peaking at 490 nm is proflavin delayed fluorescence. The shoulder peaking at 570 nm is proflavin phosphorescence. With excitation at 425 nm where only the dye absorbs, only proflavin phosphorescence can be observed under these conditions. When the ratio of proflavin concentration to that of the enzyme is increased from 0.2 to 1.0, the intensity ratio of proflavin delayed fluorescence to tryptophan phosphorescence increases by a factor of 5. (Compare Figure 2a with Figure 2b.) This result is only consistent with effectively complete binding of proflavin by the enzyme. Although the value of the association constant in water (Bernhard et al., 1966) would predict considerable free enzyme above 0 °C, our solvent does not become rigid above about −60 °C when the association would be much stronger. All subsequent measurements reported are on the 1:1 sample having the spectrum of Figure 2a. It can be seen that the tail of the tryptophan phosphorescence spectrum under the delayed fluorescence peak at 490 nm is of the order of 2% of the total intensity, and completely negligible. Therefore we can treat the emission at 485 nm as proflavin fluorescence and that at 413 nm as tryptophan phosphorescence. The luminescence decay of the complex monitored at these two wavelengths is shown in Figure 3. The logarithmic plots are normalized to the same initial intensity. These decays were analyzed at 77 K with the results given in Table I. Figure 3 represents only a few points near the beginning of the decays which were accumulated with the 1024-channel signal averager, whereas the deconvolution was done on all data points stored in the signal averager, as described in Materials and Methods. It is not possible to fit the decay at 485 nm to less than four exponential components, whereas the phosphorescence may be fit well to three exponential decays. The best evidence that the decay constants obtained from the deconvolution are significant is the agreement observed between the decay constants obtained at 413 nm and the three smallest ones obtained at 485 nm, even though the relative pre-exponential terms are very different at the two wavelengths. The triplet-singlet energy transfer model requires the phosphorescence of the tryptophans which transfer energy to have the same decay constants as the delayed fluorescence. The pre-exponential terms, however, reflect the partitioning of the triplet-state energy into the phosphorescence and delayed fluorescence channels.

Figure 4 shows the response of the phosphorescence decay and the delayed fluorescence decay to a delayed (0.4 s following closing of the exciting shutter) microwave fast passage through the $D - E$ frequency region at 1.16 K. It is observed that the initial decay of the delayed fluorescence is more rapid than that of the phosphorescence. After 0.4 s, for instance, the delayed fluorescence has decayed by 55%, while the phosphorescence has only decayed by 23%. This shows that the tryptophans which transfer energy have a shorter lifetime than those which emit phosphorescence and rules out trivial emission and reabsorption of photons as the dominant energy-transfer mechanism. The relative response

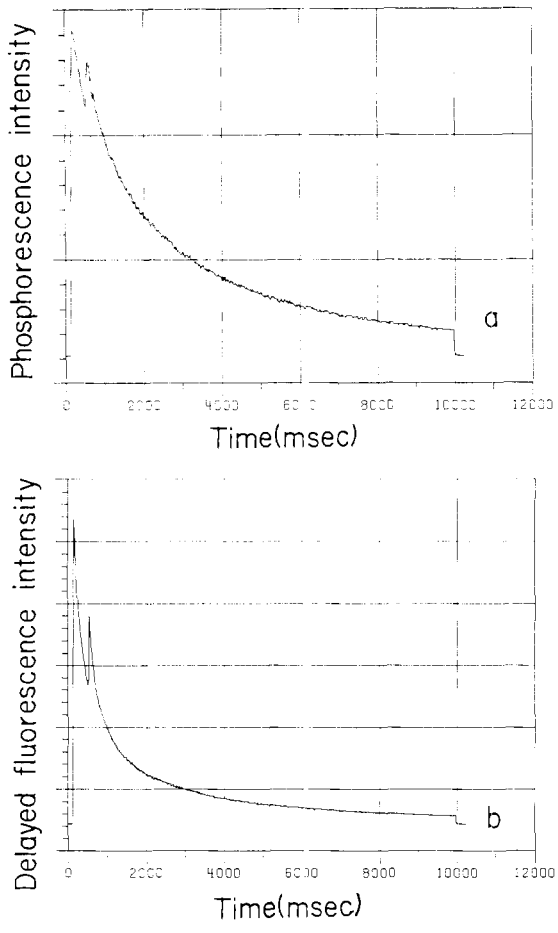

FIGURE 4: Delayed fast-passage signals observed from the enzyme-dye complex with a microwave sweep through the tryptophan ($D - E$) signal region: (a) monitoring the phosphorescence at 413 nm; (b) monitoring the proflavin delayed fluorescence at 485 nm. The short horizontal section prior to the opening of the observation shutter is the photomultiplier dark current level; T = 1.16 K. Other conditions are as in Figure 2a.

to the microwave fast passage is larger by about a factor of 3 when monitoring the delayed fluorescence than when monitoring the phosphorescence. The absolute responses differ by a much larger factor (about 17) because of the relative weakness of the phosphorescence (Figure 2a). Figure 4 thus demonstrates the ODMR signal enhancement obtainable by monitoring delayed fluorescence rather than phosphorescence. We observed similar responses for delayed fast passage through the $2E$ frequency region, but we obtained an optical response to a $D + E$ sweep only when monitoring the delayed fluorescence at 485 nm. This proves that, although T_y and T_z are not detectably phosphorescent in the complex, at least one of these sublevels transfers energy to proflavin with a sufficient transfer rate that the $T_y \leftrightarrow T_z$ transition can be seen by its effect on triplet-singlet energy transfer. It is unlikely that any of the delayed fluorescence at 485 nm is due to energy transfer from tyrosine since (a) no tyrosine ODMR signals are detectable when monitoring the delayed fluorescence decay, and (b) tyrosine phosphorescence is not even detectable in the delayed luminescence of the free enzyme, suggesting effective singlet-state quenching. In Figure 5 we show the tryptophan slow passage signals obtained by monitoring the proflavin delayed fluorescence intensity. The directly observed $D + E$ signal is a consequence of energy transfer from at least one of the "nonphosphorescent" sublevels T_y or T_z. The fact

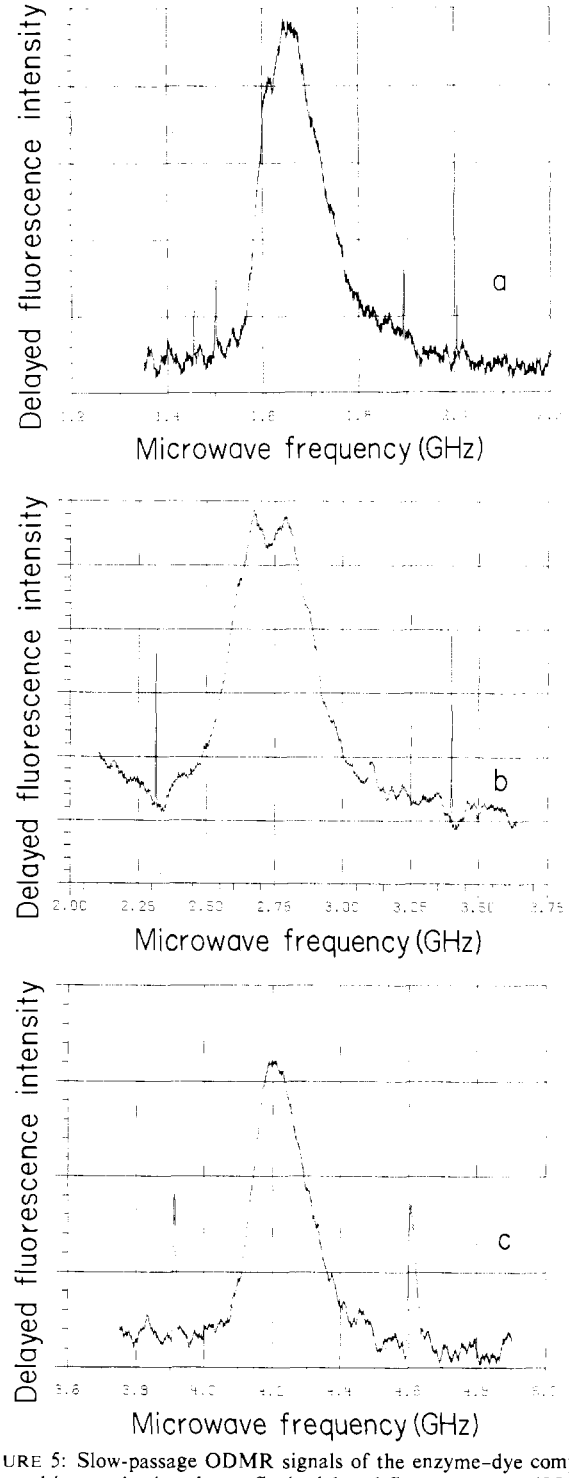

FIGURE 5: Slow-passage ODMR signals of the enzyme-dye complex observed by monitoring the proflavin delayed fluorescence at 485 nm. The microwaves are swept through the (a) ($D - E$); (b) ($2E$); and (c) ($D + E$) frequency regions of the tryptophan triplet state. Sharp vertical spikes on spectra are frequency markers. Conditions are as in Figure 2a. T = 1.16 K. Microwave scan time is 9.2 s.

that all three signals are observed implies that at least two triplet sublevels of the tryptophan residues of α-chymotrypsin transfer energy to proflavin.

Discussion

The enzyme, α-chymotrypsin, contains eight tryptophan residues in its structure, and each has the possibility of undergoing energy transfer from its triplet state to the proflavin excited singlet. The delayed fluorescence at 77 K

then would be expected to consist of a superposition of eight exponential components in the most general case. We have not attempted to fit the delayed fluorescence to this many components; indeed, we do not believe that this is possible to do unambiguously. In each of the delayed fluorescence decays which we have deconvoluted, a natural grouping into the four exponential components presented in Table I appears to occur. Triplet–singlet energy transfer rate constants are deduced by subtracting the phosphorescence decay constant of uncomplexed α-chymotrypsin from the decay constants obtained from the deconvolutions. This assumes that the decay constant of the enzyme is unaffected by binding of proflavin except for energy transfer. The triplet lifetime of tryptophan is known to be relatively insensitive to changes in local environment (see, for instance, Longworth, 1971) so we feel that the energy-transfer rate constants in Table I are not subject to significant uncertainty from this source. We think that the transfer rate constants deduced represent averages for classes of tryptophans in the enzyme–dye complex, although we are not able to give the relative numbers of tryptophan residues present in each class at this time. The reason for this is the existence of trivial energy transfer in our sample, i.e., the absorption of phosphorescence photons by the dye with reemission as fluorescence. The smallest decay constant present in the delayed fluorescence is indistinguishable from that observed in the phosphorescence decay of the enzyme, whereas it represents about 13% of the intensity of the enzyme–dye complex emission at 485 nm. Since the residual phosphorescence at this wavelength can be at most about 2% of the delayed fluorescence (Figure 2a), the long-lived component at 485 nm must be due mainly to trivial energy transfer. Further experiments are planned using thinner and more dilute samples where complications due to trivial energy transfer are expected to be absent. At present we are able to classify four types of tryptophan, of which three undergo Förster transfer to proflavin. The fastest decaying delayed luminescence component can not be observed by monitoring the phosphorescence. This is not surprising since the decay of these tryptophan residues is predicted to occur to the extent of about 95% by energy transfer to proflavin.

As the temperature is lowered sufficiently, spin–lattice relaxation becomes slower than the other rate constants (electronic decay and energy transfer), and each tryptophan triplet state will now decay as a superposition of three exponential components—the eigenvalues of the rate matrix (Zuclich et al., 1974b; Co et al., 1974). In the presence of proflavin, energy transfer in addition to the normal electronic decay will contribute to the diagonal elements of the rate matrix. Large changes in the decay kinetics are observed, in fact, when the temperature is lowered to 4.2 K and to 1.2 K which we interpret to be caused by the incipient decoupling of the sublevels due to a reduction in the spin–lattice relaxation rates. We are unable to analyze these changes to obtain the individual sublevel energy-transfer rate constants in this system because of its complexity. These rate constants could be obtained relatively easily for a simpler system consisting of a single type of triplet energy donor and singlet acceptor.

The ODMR slow-passage signals of tryptophan in enzymes typically are of the order of 100 MHz in width and, generally, do not exhibit any observable structure (von Schütz et al., 1974). The $D - E$ magnetic resonance signal of α-chymotrypsin (Figure 1a) is exceptional in that it consists of approximately nine or more partially resolved components with peaks spread out over about 100 MHz. Some structure also appears to be present in the $D + E$ EEDOR signal (Figure 1c). We can not interpret this structure at present except to say that, because of the large number of components and the wide range of their relative intensities, it is unlikely that the individual peaks correspond to the separate tryptophan sites in the enzyme. More likely, a distribution of enzyme molecular conformations makes a significant contribution to the structure. The tryptophan ODMR signals observed in the enzyme–proflavin complex by monitoring the delayed fluorescence of the dye are more typical in their large width and in their lack of fine structure. These signals are weighted heavily toward those tryptophans which are most strongly coupled to the dye molecule by Förster transfer. In the uncomplexed enzyme, the mean zero-field parameters are found to be $D/hc = 0.0985$, and $E/hc = 0.0450$ cm^{-1}. The tryptophans which transfer energy to the proflavin in the enzyme–dye complex, however, can not be fit within experimental error to a single set of zero-field parameters, D and E, since the peak frequencies of the two lowest frequency signals do not add up to give the peak frequency of the highest frequency signal. This indicates that more than one type of tryptophan (in the sense of having different D and E values) is undergoing efficient triplet–singlet transfer to proflavin, and that the contribution to each signal intensity is not the same for these types. This is quite possible since, as an example, only tryptophans which transfer energy from T_y (or T_z) can contribute to the $D + E$ signal intensity, whereas any tryptophan which transfers energy from the most radiative sublevel, T_x, contributes to the $D - E$ and $2E$ signals.

We have recently observed ODMR signals by monitoring the delayed fluorescence of proflavin bound to calf thymus DNA (Isenberg et al., 1964) while sweeping the microwaves through the thymine triplet magnetic resonance frequency regions (Dinse and Maki, 1975). We are trying to develop methods to identify sites of fluorescent dye binding in protein–nucleic acid complexes by delayed fluorescence detection of ODMR. We think that the initial results, reported here, demonstrate that this technique has promise as a structural tool for cellular material on the molecular level.

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Mutant Forms of Cytochrome P-450 Controlling Both 18- and 11β-Steroid Hydroxylation in the Rat†

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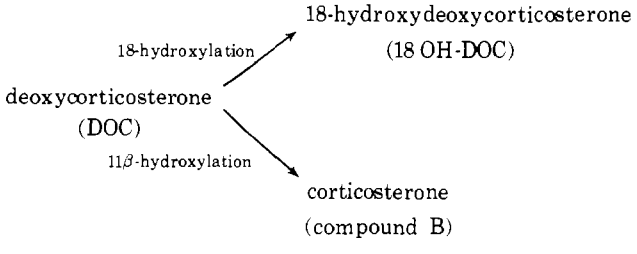
ABSTRACT: A reciprocal relationship between steroid 18- and 11β-hydroxylase activities in the salt susceptible (S) and the salt resistant (R) strains of rats was previously shown to be controlled by a single genetic locus with two alleles and inheritance by co-dominance (Rapp, J. P., and Dahl, L. K. (1972), *Endocrinology 90,* 1435). The strain specific steroidogenic patterns, characterized by the relative magnitudes of 18- and 11β-hydroxylase activities, were found to be determined by adrenal mitochondrial cytochrome P-450 particles. Carbon monoxide inhibition of 18- and 11β-hydroxylation of deoxycorticosterone in these strains showed that the CO/O_2 ratio causing 50% inhibition (i.e., Warburg's partition constant, K) was identical for 18- and 11β-hydroxylation within a strain, but different for both 18- and 11β-hydroxylation between strains. (K values were: S rats, 18-hydroxylation = 11.4 ± 1.4; S rats, 11β-hydroxylation = 11.0 ± 1.2; R rats, 18-hydroxylation = 56.4 ± 13.7; R rats, 11β-hydroxylation = 46.7 ± 11.7). This between-strain difference was unique for 18- and 11β-hydroxylation; i.e., it was not seen with cholesterol side-chain cleavage or 21-hydroxylation. Moreover, the strain-specific K values for 18- and 11β-hydroxylase and the strain-specific steroidogenic patterns due to the relative magnitudes of 18- and 11β-hydroxylase activities segregated together in an F_2 population. These data strongly suggest the same cytochrome P-450 is involved in both 18- and 11β-hydroxylation and that this cytochrome is mutated between S and R rats. K values for the reaction corticosterone → 18-hydroxycorticosterone were different between S and R strains, indicating that the mutant cytochrome was also involved in this hydroxylation, but K values for the conversion corticosterone → aldosterone were not different between strains. This was interpreted to mean that each step in the sequence corticosterone → 18-hydroxycorticosterone → aldosterone was mediated by a different cytochrome, the K value for the second step being the lower and dominating the overall reaction. It was speculated that the second step could be a second hydroxylation at position 18 to yield 18,18-dihydroxycorticosterone which would be unstable and decompose into aldosterone and water.

Rats have been selectively bred for their blood pressure response to high salt (NaCl) diet. Two strains were obtained: the susceptible or S strain and the resistant or R strain (Dahl et al., 1962). S rats respond to high salt intake with a marked increase in blood pressure, whereas R rats on the same diet show little or no blood pressure change. Recently we have described (Rapp and Dahl, 1972) a single genetic locus with two alleles inherited by co-dominance in S and R rats which controls steroidogenesis at the following branch point:



S rats show increased 18-hydroxylase activity compared with R. This increment in 18-hydroxylase activity is offset

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‡ The author notes with deep regret the death of Dr. Lewis K. Dahl, November 26, 1975.