Fast Relaxation Processes in a Protein Revealed by the Decay Kinetics of Tryptophan Fluorescence†

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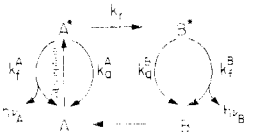
ABSTRACT: The fluorescence decay of chicken pepsinogen is not monoexponential throughout the emission spectrum. For light emitted at the long wavelength region of the fluorescence spectrum, the decay can be described by two exponential terms, one of them exhibiting a negative amplitude. This behavior shows that in this region of the spectrum the fluorescence builds up before it decays, indicating that the electronically excited species involved has been created during the fluorescence lifetime. For a variety of reasons it seems very unlikely that this emitting species was formed by energy transfer from other entities in the protein. The buildup of the fluorescence at the red edge of the spectrum prior to emission thus reflects a genuine relaxation process in the protein molecule in the nanosecond time scale, the excited chromophore shifting its emission spectrum to the red in the course of the relaxation process. The reaction involved in the relaxation may be a nonspecific orientation of various groups around the excited chromophore or a formation of a more specific excited state complex, *i.e.*, an exciplex. This nanosecond relaxation process is conformation dependent and disappears upon denaturation. Similarly, chicken pepsin at neutral pH fails to show it. This seems to be the first case in which a relaxation process in the nanosecond time range has been demonstrated with a native chromophore in a protein and complements the observations of Brand and Gohlke and of Lakowicz and Weber of other nanosecond relaxation processes in proteins. Fast relaxation reactions demonstrated in the present study for tryptophans are not necessarily limited to indole side chains, except that these residues permit the study of the relaxation processes by perturbation of interactions with the environment through electronic excitation.

The fluorescence emitted by molecules in condensed phases is invariably shifted to longer wavelengths relative to the absorption spectrum. The larger the interaction between the excited chromophore with its environment the greater the shift of the fluorescence band to the red. This behavior of fluorescence spectra is due to relaxation processes which the excited molecule undergoes prior to the emission process, the rate of relaxation being usually much faster than the rate of light emission. The processes which take place in the excited state may be of a variety of kinds: dissipation of vibrational energy or change of conformation of the electronically excited molecules, interactions with surrounding solvent molecules, association or dissociation reactions in the excited state, or processes of fast energy transfer from one chromophoric group to another in the system studied. Part of the energy of the excited molecules is lost in these processes and the photon emitted subsequently is left with less energy to carry; the emitted light is thus of longer wavelength.

In all cases in which the reactions in the excited state are much faster than the rate of light emission, the fluorescence collected at the various wavelengths of the spectrum decays monotonously with time. Under certain circumstances some of the relaxation processes are not fast compared to the lifetime of the excited state. Such a situation is disclosed by buildup of the fluorescence intensity prior to the decay at some region of the emission spectrum. Examples of this kind were reported when the electronically excited molecule undergoes association reactions (Speed and Selinger, 1969) or ionization reactions (Loken *et al.*, 1972) or when it is embedded in a highly viscous medium (Ware *et al.*, 1971; Brand and Gohlke, 1971). It may be recalled that the lifetime of the first singlet excited state, which is the one that is usually involved in the fluorescence process, is of the order of 1–100 nsec. The study of the decay kinetics at different wavelengths or of the behavior of the emission spectrum at various times after excitation may thus serve as a powerful tool in the investigation of excited-state processes in the nanosecond time range.

While relaxation processes may follow very complicated kinetics depending on the exact details of the reactions involved, it is useful to assume a simple scheme for the chain of reactions which follows the electronic excitation in order to gain qualitative understanding of the events that take place. The following scheme (eq 1) has been adopted, where



(1)

A* is the excited species formed immediately upon light absorption by the absorbing molecule. It is then converted with a rate constant k_r into a second excited species B*. A* can also fluoresce or decay nonradiatively with rate constants k_f^A and k_q^A, respectively. B* may undergo similar reaction with respective rate constant k_f^B and k_q^B. Since energy is dissipated on the conversion of A* into B*, the light emitted by B* will be red-shifted relative to that emitted by A*. The fluorescence at the red-edge of the emission spectrum, where the emission from B* is dominant, should thus show a buildup prior to decay. For the simple scheme presented in eq 1 the analytical expression for the decay of the concentration of A* and B* is given by

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$$[A^*] = [A^*]_0 e^{-\gamma_1 t}$$
$$[B^*] = \frac{k_r[A^*]_0}{\gamma_1 - \gamma_2}(e^{-\gamma_2 t} - e^{-\gamma_1 t}) \quad (2)$$

where $[A^*]_0$ is the concentration of A* at $t = 0$, and $\gamma_1 = k_f^A + k_q^A + k_r$ and $\gamma_2 = k_f^B + k_q^B$. Equation 2 is of the same form as the equations given by Loken *et al.* (1972) for ionization reactions in the excited state when the proton transfer is not reversible. Under real circumstances, eq 2 cannot be expected to hold exactly since the scheme presented in eq 1 is probably an over-simplification, and it is experimentally difficult to isolate the emission due to B* alone. However, the appearance of an exponent with a negative amplitude in the expression obtained for the fluorescence decay at the red-edge of the emission spectrum shows that a relaxation process of one kind or another has taken place in the system studied.

The above approach has been applied in the study to be reported below to the detection and examination of nanosecond processes which take place in protein molecules. The intrinsic fluorescence of proteins due to their tryptophan residues was measured at different spectral ranges and analyzed for its time course after excitation. In the case of the protein chicken pepsinogen a buildup of the fluorescence intensity at the red edge of the emission spectrum was demonstrated, indicating that relaxation processes do indeed take place in this protein with a lifetime of less than a nanosecond.

Experimental Section

Materials. Highly purified chicken pepsinogen and chicken pepsin have been kindly donated by Dr. Zvi Bohak of the Department of Biophysics, the Weizmann Institute of Science. The procedures for purification and characterization of these proteins were described elsewhere (Bohak, 1969). Most of the measurements were carried out at pH 7.1 (0.1 M phosphate buffer in doubly distilled water) using freshly prepared solutions. For measurements at different pH values the solution was titrated in the cuvet by addition of either 1 N HCl or 1 N NaCl, the pH being monitored by a pH meter (Radiometer, Copenhagen). Purified *N*-acetyl-L-tryptophanamide and *N*-methylpyridinium perchlorate were a generous gift from Dr. M. Shinitzky. Solvents used were of Spectrograde quality; lack of fluorescent impurities was tested in every case by using the very sensitive decay fluorimeter.

Methods. Protein concentrations were determined by the absorbance at 280 nm using absorption coefficients of 1.26 and 1.46 mg^{-1} cm^2 for the pepsinogen and pepsin, respectively (Bohak, 1969). Protein concentrations used were always less than 3×10^{-5} M. Unless otherwise stated all measurements were carried out at a temperature of 23 ± 2°.

Absorption spectra were measured with a Zeiss Model PMQ II or with a Cary 15 spectrophotometer. Corrected fluorescence spectra were obtained with a Turner 210 spectrofluorimeter using cells of 1-cm optical path, the optical density of the solution being less than 0.1 at the excitation wavelength. Fluorescence polarization spectra[1] were obtained with an instrument of the type described by Weber and Bablouzian (1966), built by Dr. M. Shinitzky, the Weizmann Institute of Science. The fluorescence was excited by linearly polarized light. Special filters were used to isolate either the blue or the red part of the emission spectrum, Kodak 18A filter for the blue region, and a Schott KV-380 filter for the red region (see Figure 3). For the fluorescence polarization studies the proteins were first dissolved in a few drops of water and then diluted with a large excess of propylene glycol, and cooled down to −50°.

The instrument for the measurement of the fluorescence decay was of the type described by Hundley *et al.* (1967). The excitation wavelength was selected by a Jarrel-Ash double monochromator; the wavelength band width was 2–6 nm. The emission wavelength was monitored by using various filters which have negligible fluorescence (see Figure 3). A useful modification has been introduced in our laboratory to overcome problems due to drift which are associated with the repeated averaging procedures necessary for measurements of fluorescence decay (Hazan *et al.*, 1974). In the modified instrument the fluorescent solution and a scattering suspension are introduced alternatively and repeatedly into the light path of the instrument, and the signals from each solution were collected separately in the two halves of a multichannel analyzer.

Data Analysis. The fluorescence decay data were analyzed by the method of nonlinear least squares (Grinvald and Steinberg, 1974). The decay kinetics were assumed to be monoexponential or multiexponential functions of the type $i(t) = \Sigma_i \alpha_i \exp(t/\tau_i)$, where $i(t)$ is the assumed decay function, and α_i and τ_i are the amplitude and lifetime, respectively, of the ith exponent. The amplitudes and the lifetimes are taken as free parameters to be estimated by best fit of the data. The assumed decay function $i(t)$ was convoluted with the profile of the excitation lamp, $G(t)$, using the convolution integral $F_c(t) = {}_0\int^t G(t - s)i(s)ds$. The result $F_c(t)$ was then compared with the experimental decay $F(t)$. A sophisticated computer program was then used to find the parameters which yield the best fit between the calculated and experimental decay curves of the fluorescence by minimizing the RMS, *i.e.*, the root

$$\text{RMS} = \left\{\frac{1}{n}\sum_{i=1}^{n}[F_c(t_i) - F(t_i)]^2\right\}^{1/2}$$

mean square of the deviations between the calculated and experimental curves. The nonnormalized autocorrelation function of the deviations, $R(t_j)$,[2] is also included as a convenient qualitative criterion for the presence of systematic deviations between the calculated and measured fluorescence decay (Grinvald and Steinberg, 1974). Part of the data was analyzed by the method of moments (Isenberg and Dyson, 1969), and the results are very similar to those obtained by the method of nonlinear least squares.

If the decay kinetics is described by a multiexponential decay function, the parameters calculated for this function are very much affected by the amount of noise and systematic error associated with the measurements (Grinvald and Steinberg, 1974). It is therefore most important to check the quality of the experimental data. We have tested the instrumental set-up by using a fluorescence decay standard,

[1] The fluorescence polarization p is defined as $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, where $I_\parallel$ and $I_\perp$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular, respectively, to the direction of polarization of the exciting light.

[2] $R(t_j) = 1/m\ \Sigma_{i=1}^{m} \Delta_i \Delta_{i+j}$, where $\Delta_i = F(t_i) - F_c(t_i)$, note that $\text{RMS} = (R(t_0))^{1/2}$, *i.e.*, the square of the intercept of the nonnormalized autocorrelation function yields the RMS. The nonnormalized autocorrelation function was found to serve as a better visual criterion for the quality of the fit between $F(t)$ and $F_c(t)$ than the normalized autocorrelation function used previously (Grinvald and Steinberg, 1974).

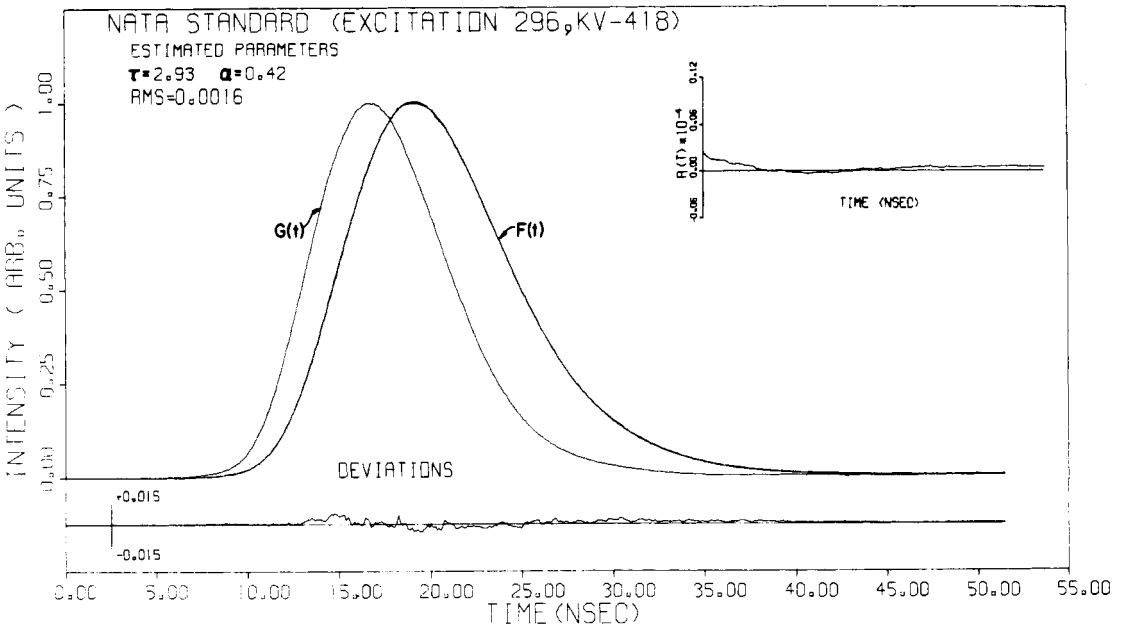

FIGURE 1: Test of the fluorescence decay instrument by *N*-acetyl-L-tryptophanamide, NATA, used as a standard. Analysis of 5×10^{-5} M NATA, in 0.1 M phosphate buffer (pH 7.1). Excitation wavelength, 296 nm. Only the red edge of the emission is collected by transmission through the KV-418 filter. $G(t)$, excitation light profile. $F(t)$, two superimposed curves, the noisy curve is the experimental fluorescence decay curve, the smooth curve is the calculated fluorescence decay assuming a monoexponential decay function for the standard substance. Noisy curve at the bottom of the figure is the trace of the deviations between the experimental and the calculated curves. Insert: the nonnormalized autocorrelation function of the deviations. The fit to a single exponent is excellent as seen by the very low RMS (16×10^{-4}) and the shape of the nonnormalized autocorrelation function.

i.e., a pure substance which is expected to exhibit monoexponential decay. The substance chosen was highly purified *N*-acetyl-L-tryptophanamide, NATA.[3] The results of a typical experiment for such a test with the standard are presented in Figure 1. The data could be fitted to monoexponential decay kinetics with a very low standard deviation of 1.6×10^{-3}. (The maximum of the fluorescence is arbitrarily normalized to unity.) Inspection of the trace of the deviations and of the nonnormalized autocorrelation of the deviations (see Figure 1) shows that the data contain very low random noise and are almost free of systematic error. Thus, the deviations at the peak of the signal are of the order of 3×10^{-3} of the signal, which should be compared with a corresponding value of $5\text{–}10 \times 10^{-3}$ recently reported for data collected by the monophoton counting technique (Dyson and Isenberg, 1971). This low level of random noise and of systematic error shows that with due care the sampling scope technique of measuring nanosecond decay can achieve an accuracy and precision that is competitive with that obtained by other methods, including the monophoton counting technique (A. Grinvald, to be published). Special precautions must be taken to prevent stray light, fluorescence of filters, or fluorescence of contaminants. Each new set of experiments with a protein solution was therefore accompanied with a measurement of the fluorescence decay of the standard substance under identical conditions to check the quality of the data. Whenever the fluorescence decay of a protein could not be fitted to a monoexponential decay function, more exponents were added to the assumed decay function till the standard deviation between the calculated and experimental data was of similar magnitude to that found for the standard substance, NATA, assuming for the latter a monoexponential decay function.

The parameters obtained by any method of analysis are bound to have a range of uncertainty depending upon the level of the random noise (assuming an ideal instrument) and the correlations which exist among the parameters of the decay function. We have presented a general statistical procedure for estimation of the range of stochastic error in the values of the parameters of any decay function (Grinvald and Steinberg, 1974). Following this procedure, for the decay parameters of the red-edge emission of chicken pepsinogen excited at 296, we got the following results: $7.27 < \tau_1 < 7.29$; $0.646 < \tau_2 < 0.726$; $0.260 < \alpha_1 < 0.261$; $-0.114 < \alpha_2 < -0.101$, thus demonstrating a negligible stochastic error with this set of parameters. Similar ranges for the stochastic errors of the decay parameters were obtained for other sets of parameters assumed for the decay function which describe the red edge of the emission. The percentage error is always greater for τ_2 and α_2. Note that α_2 and τ_2 are well correlated, *i.e.*, if the error associated with τ_2 is positive then α_2 should decrease and *vice versa*. For the decay parameters obtained for the blue region of the emission, τ_1 and α_1 have negligible error and τ_2 and α_2 may vary by less than 10%. It can be shown by computer simulations that relaxation processes with lifetimes as short as 10 psec should be detectable by deviations from monoexponential decay; however, minor amounts of contaminants with fast decaying fluorescence or scattered light will then mask the exponent with the negative amplitude.

Figure 1 shows that our instrument still has a rather small systematic error which is easily detected by the nonnormalized autocorrelation function. If we correct our data for this systematic error it will influence only the values of the parameters of the negative exponent, increasing them by about 10%. Errors in τ values due to timing calibration of our instrument are less than 2%.

Results

The absorption and emission spectra of chicken pepsino-

[3] Abbreviation used is: NATA, N-acetyl-L-tryptophanamide.

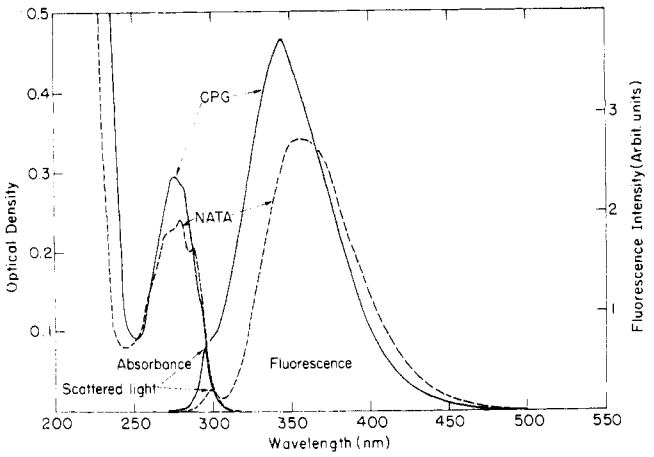

FIGURE 2: Absorption and emission spectra of chicken pepsinogen, CPG, and *N*-acetyl-L-tryptophanamide, NATA, in 0.1 M phosphate buffer (pH 7.1). Excitation wavelength, 296 nm. CPG has a quantum yield which is 1.3-fold greater than that of NATA.

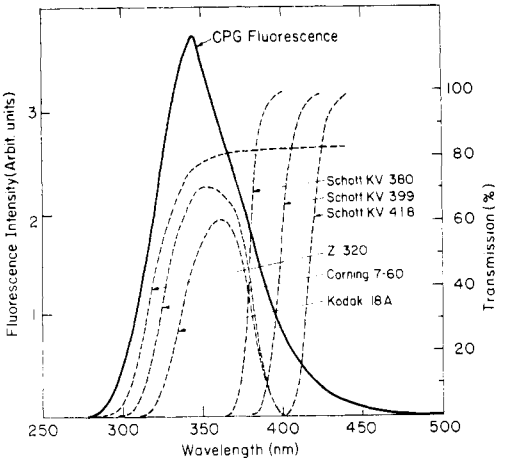

FIGURE 3: Transmission curves of the filters which were used to isolate different regions of the emission spectra. The emission spectrum of CPG is included.

gen are presented in Figure 2. For comparison, the respective spectra of *N*-acetyl-L-tryptophanamide, NATA, are included in the same figure. The absorption spectrum of the protein lacks the details observed for the model compound. It should be noted that there are five tryptophan residues and 24 tyrosine residues per protein molecule (mol wt 43,200) (Bohak, 1969). The overlap of the spectra of the various residues probably contributes to the lack of details in the absorption spectrum. The emission spectrum is shifted to shorter wavelengths relative to that of NATA in water, indicating that at least some of the tryptophan residues are in a less polar environment than water (Walker *et al.*, 1967; Longworth, 1971). The fluorescence spectrum of the protein was obtained by excitation at 280, 295, and 305 nm and was practically independent on excitation wavelength. In view of the fact that tyrosine residues absorb differently at these wavelengths, it follows that the fluorescence of chicken pepsinogen is due to the tryptophan residues in the molecule with no detectable contribution from the tryosine residues.

The kinetics of the decay of the fluorescence of chicken pepsinogen was studied using different filters to isolate various portions of the emission spectrum. The transmission spectra of the filters used are presented in Figure 3. Thus, the filters 7-60 and 18-A transmit the short wavelength portion of the emission spectrum, while the filter KV-418 transmits the long wavelength tail of the emission spectrum exceeding 418 nm. A typical fluorescence decay experiment is presented in Figure 4. The profiles of the intensities of the excitation lamp and of the fluorescence are related to each other by the convolution integral, and yield by least-squares fitting the sum of exponentials that characterize the fluorescence decay of the protein. The decay functions of the fluorescence obtained at the spectral regions transmitted by the various filters described in Figure 3 are presented in Table I. The decay curves are definitely not monoexponential. At the short wavelength portion of the emission spectrum two lifetimes of about 7 and 1.2 nsec are observed with a ratio of the amplitudes of about 1.5:1. At the long wavelength portion of the spectrum an exponent with a negative preexponent is clearly indicated, with a lifetime of about 0.7 nsec, accompanied by a regular exponent with a lifetime of about 7 nsec. As explained above, this negative amplitude is clear-cut proof that the light emitted at the red edge of the fluorescence spectrum builds up before it decays and reflects the fact that the species responsible for emission at this spectral region is created with a reaction lifetime of the order of a nanosecond from the primary absorbing species.

The above experiments were repeated with excitation light of different wavelengths. The results are summarized in Table II. It is seen from this table that an exponent with a negative amplitude is obtained at the red edge of the emission spectrum regardless of the excitation wavelength. These results clearly demonstrate that the above phenomenon cannot be explained exclusively by absorption of excitation light by tyrosine residues and subsequent transfer of energy to tryptophans, since tyrosines show practically no absorption at 305 nm, while excitation at this wavelength still results in a negative preexponent at the red edge of the emission spectrum. It may be noted that the negative ampli-

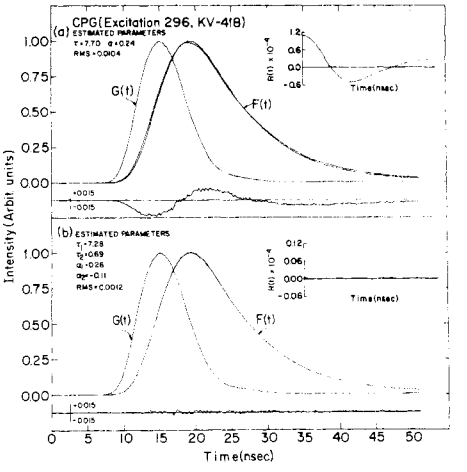

FIGURE 4: Analysis of the fluorescence decay of CPG at the red edge of the emission spectrum (filter, KV-418). Protein concentration, 2×10^{-5} M; experimental conditions are the same as in Figure 1. (a) Analysis assuming a monoexponential decay. The high value of the RMS, the nonrandom distribution of the deviations, and the shape of the nonnormalized autocorrelation function show that the decay function is definitely not monoexponential. (b) Analysis assuming biexponential decay. The low RMS value (12×10^{-4}), the random distribution of the deviations, and the shape of the nonnormalized autocorrelation function (note: the scale is tenfold expanded relative to (a)) show that the fit is excellent. The parameters of the decay function are $\tau_1 = 7.25$ nsec, $\tau_2 = 0.68$ nsec, and $\alpha_1/\alpha_2 = -2.3$.

TABLE I: Values of the Decay Parameters of the Fluorescence of Chicken Pepsinogen at Different Regions of the Fluorescence Emission.[a]

	One-Component Analysis[c]		Two-Components Analysis[c]				
Emission Filter[b]	$\langle\tau\rangle$ (nsec)	RMS[d] ($\times 10^{-4}$)	τ_1 (nsec)	τ_2 (nsec)	α_1	α_2	RMS[d] ($\times 10^{-4}$)
Corning 7-60	6.1	181	7.1	1.2	0.60	0.40	15
Kodak 18A	6.4	130	7.1	1.4	0.70	0.30	16
	(7.0)	(149)	(7.9)	(1.4)	(0.70)	(0.30)	17
Glass Z320	6.6	103	7.1	1.4	0.76	0.24	17
Schott KV380	7.5	85	7.3	0.7	0.81	−0.19	10
	(8.3)	(84)	(8.0)	(0.6)	(0.77)	(−0.23)	17
Schott KV399	7.6	91	7.3	0.6	0.75	−0.25	13
Schott KV418	7.7	104	7.3	0.7	0.70	−0.30	11
	(8.5)	(128)	(8.0)	(0.7)	(0.72)	(−0.28)	17

[a] For the range of error of these parameters see the paragraph on data analysis in the Experimental Section. Protein concentration 2×10^{-5} M in 0.1 M phosphate buffer (pH 7.1). Excitation wavelength, 296 nm. [b] The transmission curves of the different filters are presented in Figure 3. [c] Values in brackets are obtained in D_2O solutions. [d] The RMS obtained upon fitting the decay of NATA to a single component is 10×10^{-4}–18×10^{-4} depending on how long the experiment was carried on. Thus, all the decay kinetics quoted in this table are definitely not monoexponential but can be fitted to biexponential functions within experimental accuracy. The true decay functions may be more complex, but the quality of the data does not justify further reliable refinement.

tude diminishes somewhat upon excitation at longer wavelengths. This is probably due to excitation of partially relaxed states when one applies photons of low energy to induce transition to the excited state. Similar diminishing of the negative amplitude upon excitation at the red tail of the absorption band was also observed in low molecular weight model systems which exhibit time-resolved spectra (A. Grinvald and I. Z. Steinberg, to be published).

An exponent with a negative amplitude characterizes the red edge of the emission spectrum of chicken pepsinogen at pH 7 and 9, as well as at pH 2.9 (Table III), although at the latter pH the peptide which is removed from the protein on conversion to chicken pepsin is already split off (Bohak, 1973). The term with the negative preexponent disappears at pH 10.8, at which the protein denaturation just begins. The fluorescence of chicken pepsin at neutral pH is characterized by a decay that is practically monoexponential. The above results are summarized in Table III. It may be noted that the exponent with the negative amplitude persists on lowering the temperature to 4° or raising it to 45°. The exponent with the long lifetime is somewhat affected by the temperature, the corresponding τ decreasing with the rise of temperature.

The fluorescence spectra of chicken pepsinogen in the presence of 0.1 and 0.2 M *N*-methylpyridinium perchlorate are presented in Figure 5. Pyridinium ions are known to quench the fluorescence of tryptophan (Shinitzky and Katchalski, 1968); indeed, the quantum yield of the protein is decreased in the presence of pyridinium ions by a factor of 7. It is of interest that the remaining fluorescence is appreciably blue shifted (by about 10 nm) relative to the emission spectrum of the protein in water. Assuming a dynamic quenching of the type described by Lehrer (1971), it is thus shown that the fluorescence of the protein is composed of contribution from different tryptophan residues, each with a somewhat different emission spectrum, and that the pyridinium ions quench preferentially those residues whose emission spectrum is shifted to the red. It may be noted that the fluorescence of chicken pepsinogen is enhanced by about 10%, and shifted to the red by 1 nm, when dissolved

TABLE II: Values of the Decay Parameters of the Red-Edge Emission of Chicken Pepsinogen as a Function of the Exciting Wavelength.[a]

Exciting Wavelength (nm)	One-Component Analysis		Two-Components Analysis				
	$\langle\tau\rangle$ (nsec)	RMS ($\times 10^{-4}$)	τ_1 (nsec)	τ_2 (nsec)	α_1	α_2	RMS ($\times 10^{-4}$)
250	7.0	65	6.8	0.3	0.75	−0.25	22
276	7.4	100	7.1	0.5	0.66	−0.33	11
280	7.4	101	7.1	0.5	0.66	−0.33	14
296	7.5	85	7.3	0.7	0.81	−0.19	13
305	7.4	34	7.3	0.4	0.84	−0.16	20

[a] For the range of error of these parameters see the paragraph on data analysis in the Experimental Section. The emission filter used was KV-380, see Figure 3. Protein concentration 2×10^{-5} M at 0.1 M phosphate buffer (pH 7.1). For excitation at 305 nm the concentration was 4×10^{-5} M.

TABLE III: Values of the Decay Parameters of the Red-Edge Fluorescence of Chicken Pepsinogen at Various Conditions.[a]

Sample	One-Component Analysis		Two-Components Analysis				
	$\langle\tau\rangle$ (nsec)	RMS ($\times 10^{-4}$)	τ_1 (nsec)	τ_2 (nsec)	α_1	α_2	RMS ($\times 10^{-4}$)
Pepsinogen, pH 2.9 (pepsin)	6.8	50	6.7	0.4	0.77	−0.23	15
Pepsinogen, pH 6	7.4	61	7.2	0.4	0.76	−0.24	13
Pepsinogen, pH 7.1	7.5	85	7.3	0.7	0.81	−0.19	13
Pepsin, pH 7.1[b]	7.1	24	Almost monoexponential no negative amplitude				
Pepsinogen, pH 9	7.6	82	7.3	0.5	0.73	−0.27	10
Pepsinogen, pH 10.8[c]	6.3	26	Almost monoexponential no negative amplitude				
Pepsinogen, D_2O, pH 7.1	8.3	84	8.0	0.6	0.77	−0.23	17
Pepsinogen, +0.1 M pyridinium, pH 7.1	2.7	36	2.5	0.3	0.75	−0.25	11
Pepsinogen, pH 7.1, 45°	5.9	61	5.7	0.6	0.79	−0.21	14
Pepsinogen, pH 7.1, 4°	8.2	58	8.0	0.4	0.71	−0.29	22
Pepsinogen, glycerol, pH, 7.1[d]	7.2	83	6.8	0.7	0.74	−0.26	17

[a] For the range of error of these parameters see the paragraph about data analysis in the Experimental Section. KV-380 filter was used, see Figure 3. Protein concentration, 2×10^{-5} M. [b] The same results were obtained either when purified pepsin was brought to this pH, or pepsinogen was activated at lower pH and then brought again to neutral pH. [c] At this pH the pepsinogen just begin to denature. [d] The protein was first dissolved with a few drops of buffer and then diluted with a large excess of glycerol.

in D_2O instead of H_2O. In this case the fluorescence of the red tryptophans is preferentially enhanced. These experiments demonstrate that the light emitted from this protein is heterogeneous and that different tryptophan residues contribute differently at various regions of the emission spectrum. Furthermore, there is not a complete exchange of excitation energy among the tryptophan residues in the molecule. The fluorescence of NATA is quenched by pyridinium ions and is enhanced by D_2O. As expected, there is no shift in the emission spectrum in this case. The results summarized in Table III demonstrate that the fluorescence decay of chicken pepsinogen at the red edge of the spectrum exhibits an exponent with a negative amplitude also in 0.1 M *N*-methylpyridinium perchlorate solution. A comparison between the lifetime measurements in D_2O and H_2O (Table I) shows that D_2O affects only the long lifetime and has no effect on the amplitudes or the short lifetime.

The excitation spectrum of the fluorescence of chicken pepsinogen was measured for different emission wavelengths. Identical spectra, with the maximum at 280 nm, were obtained when the emitted light was monitored at 320, 395, or 410 nm. Since the different tryptophans in the molecule contribute differently at the different wavelengths, the above results indicate that the various tryptophans have very similar absorption spectra. These results also indicate that tyrosine residues do not transfer excitation energy to tryptophan residues, since such transfer is very unlikely to take place equally to all tryptophans; the tyrosine would thus affect differently the excitation spectra associated with the emission of the different tryptophan residues if Tyr → Trp energy transfer takes place, contrary to the observed excitation spectra.

The linear polarization of chicken pepsinogen for light emitted at the blue region and the red region of the fluorescence spectrum is presented in Figure 6. For comparison, the linear polarization of *N*-acetyl-L-tryptophanamide in propylene glycol at −50° is shown in the same figure. As is seen from the above figure, the fluorescence polarization of the protein and of the low molecular weight compound at the red edge of the absorption spectrum are similar in magnitude. This result is in accord with the findings of Weber and Shinitzky (1970) that transfer of electronic excitation energy between like chromophores, including tryptophans, ceases upon excitation at the red edge of the absorption spectrum. It may be noted that somewhat different values are observed for the polarization when the fluorescence is detected at the short edge or long edge of the fluorescence spectrum.

Discussion

The results presented above (Figure 3 and Tables I and II) clearly show that the fluorescence emitted by the trypto-

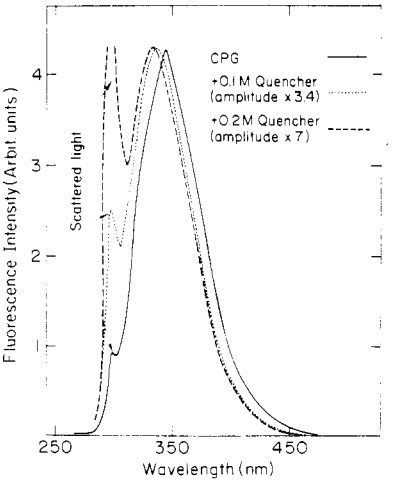

FIGURE 5: The quenching of the fluorescence of chicken pepsinogen, CPG, by *N*-methylpyridinium perchlorate. Protein concentration, 2×10^{-5} M, in 0.1 M phosphate buffer (pH 7.1). Excitation wavelength, 295 nm.

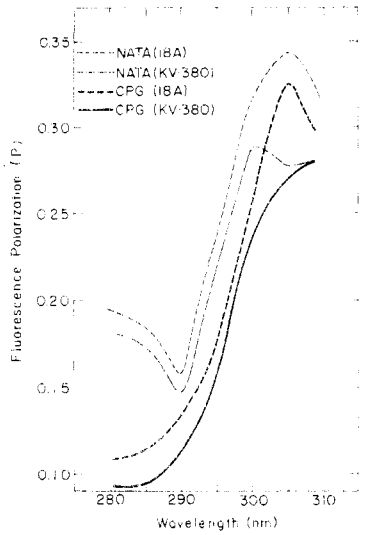

FIGURE 6: Linear polarization spectra of chicken pepsinogen, CPG, and of *N*-acetyl-L-tryptophanamide, NATA. Concentration of both substances, 3×10^{-5} M in 95% propylene glycol. Temperature, −50°.

phan residues of chicken pepsinogen does not decay monoexponentially and that for the light emitted at the red edge of the fluorescence spectrum an exponent with a negative amplitude appears in the decay kinetics. Such kinetics shows that the species responsible for the emission at the long wavelength region of the spectrum builds up in concentration with a finite rate constant of the order of 10^9 sec^{-1} following the absorption of the light. For the sake of simplicity let us adopt the simplified scheme presented in eq 1 and described by eq 2. The intensity $I(\lambda)$ obtained at wavelength λ after time t following excitation will thus be given by

$$I(\lambda) = c(I_A(\lambda)[A^*] + I_B(\lambda)[B^*]) = c[A^*]_0 \left\{ \left(I_A(\lambda) - I_B(\lambda)\left(\frac{k_r}{\gamma_1 - \gamma_2}\right)\right) e^{-\gamma_1 t} + I_B(\lambda)\left(\frac{k_r}{\gamma_1 - \gamma_2}\right) e^{-\gamma_2 t} \right\} \quad (3)$$

where $I_A(\lambda)$ and $I_B(\lambda)$ are the intensities of emission per unit concentration of A* and B*, respectively, at wavelength λ, and c is a proportionality factor dependent on the experimental set-up used. Equation 3 demonstrates that for the model system discussed an exponent with a negative preexponent will appear in the decay kinetics of the fluorescence for such wavelength at which $I_B(\lambda)k_r/(\gamma_1 - \gamma_2) > I_A(\lambda)$. This condition is fulfilled in the spectral range at which the emission of [B*] predominates. At the range at which the emission of [A*] predominates the kinetics will be described by a regular sum of exponentials.

While the model represented by eq 3 is useful to gain a qualitative understanding of the requirements for the occurrence of negative preexponents in fluorescence decay kinetics, it is necessarily an oversimplified description of real cases. This is specially so in the case of the fluorescence of proteins. If the negative amplitude stems from vibrational relaxation of the excited molecule or from interactions with the surrounding solvent molecules, B* cannot be defined as a single species, and a tandem of intermediate species may be formed between the one that absorbs the photon and the one that emits it. Furthermore, in the case of proteins, if a few tryptophan residues are present in each molecule they may have different decay times (Yashinsky, 1972) which add up together in addition to the kinetics described by eq 3. If some of the tryptophan residues have a regular exponential decay with a lifetime comparable to γ_1^{-1} (see eq 3), they may partially or totally mask the appearance of the negative amplitude. This is possibly one of the reasons why a fluorescence decay kinetics with a preexponent was not found to be a common occurrence. Among the proteins tested (staphlococcal nuclease, *Pseudomonas* azurin, naja naja toxin, human serum albumin, bovine seruman albumin, hen egg-white lysozyme, human lysozyme, papein, procine pepsinogen, procine pepsin) only chicken pepsinogen was found to show it unequivocally. Most of these proteins have more than one tryptophan residue per molecule, and human serum albumin naja naja toxin, and azurin, though possessing a single tryptophan per molecule exhibit multiexponential decay possibly due to heterogeneity in molecular structure (de-Lauder and Wahl, 1971; Hazan, 1973; Grinvald, Schlessinger, Pecht, and Steinberg, in preparation).

The negative amplitudes in the fluorescence decay kinetics of chicken pepsinogen appear under a variety of conditions. Thus, it persists at acid pH, at which the peptide bond responsible for the pepsinogen → pepsin conversion is already cleaved, or in D_2O, or upon partial quenching of fluorescence by pyridinium ions. It is of interest, however, that the negative amplitude disappears in chicken pepsin at neutral pH. Thus, the relaxation process responsible for the negative amplitude in pepsinogen apparently does not take place in pepsin, reflecting a difference in the environment of the pertinent indole side chain (or chains) in the two proteins.

The fluorescence properties of the tryptophan residue (or residues) which exhibit the relaxation process in the excited state are quite interesting. The fluorescence is at relatively long wavelength, which would indicate that the emitting indole residues are in a polar environment. It seems to be exposed, at least to some extent, to the solvent, since it is affected by the presence of D_2O or pyridinium ions. The decay lifetime is quite long, indicating that the residues under discussion have a rather high quantum yield. On the other hand, the relaxation processes associated with these residues as disclosed by the fluorescence decay kinetics are apparently not due to relaxation of water molecules around the chromophores, since very similar relaxation kinetics was observed in glycerol solution (see Table III), a solvent which is so distinctly different from water with respect to the relaxation properties of its molecules. It thus seems that although exposed to the solvent these indole side chains still have significant interactions with the rest of the protein molecule.

The nature of the process which leads to the formation of a species B* responsible for the special kinetics of the fluorescence of chicken pepsinogen deserves attention. It may be discussed in reference to two possible classes of processes: (a) relaxation processes, in which the excited tryptophan residue undergoes interaction with its environment in the nanosecond time range; (b) energy transfer among the chromophores in the protein. The possibility that the phenomenon is due to energy transfer from tyrosine residues to tryptophan residues may be dismissed immediately, since the negative preexponent in the fluorescence decay persists upon excitation at 305 nm, at which wavelength the tyrosine residues practically do not absorb any light. The possibility that the phenomenon is due to energy transfer from one tryptophan to another (*i.e.*, that A* and B* in eq 3 are a donor tryptophan residue and an acceptor tryptophan residue, respectively), seems to be very unlikely for a variety of

reasons. Weber and Shinitzky (1970) have shown that energy transfer among like residues stops upon excitation at the red edge of their absorption spectrum. The negative preexponent persisted, however, upon excitation at the red edge of the absorption spectrum of the protein (see Table II). The linear polarization spectrum shown in Figure 6 is in agreement with this argument: upon increase of wavelength the value of the linear polarization approached that of the low molecular weight tryptophan compound, NATA, at high dilutions, at which inter-tryptophan energy transfer is not expected to take place. It is pertinent to note that if inter-tryptophan energy transfer is the cause for the special fluorescence kinetics observed for chicken pepsinogen, it has to be a unilateral transfer process. If A* and B* in eq 3 are two tryptophan residues which can equally transfer energy to each other, their fluorescence decay curve cannot contain an exponent with a negative amplitude (Hazan, 1973). For unilateral energy transfer to take place between like chromophores, special requirements have to be imposed on their spectral properties and on their quantum yields (Steinberg, 1971). The probability of the transfer of electronic excitation energy per unit time from a donor molecule to an acceptor molecule, for a defined intermolecular distance, orientation, and medium, is proportional to the quantum yield of the donor (in the absence of acceptor) and the overlap integral defined as

$$\int_0^\infty [f_D(\bar{\nu})\epsilon_A(\bar{\nu})/\bar{\nu}^4]d\bar{\nu}$$

where $f_D(\bar{\nu})$ is the normalized fluorescences intensity of the donor at wave number $\bar{\nu}$ and $\epsilon_A(\bar{\nu})$ is the absorption coefficient of the acceptor at $\bar{\nu}$. The spectral properties of the individual tryptophan residue in the protein are of course not known quantitatively, but to get a pronounced unilateral transfer of excitation energy between two tryptophan residues, their fluorescence properties should be dramatically different. While this is not impossible, it is very unlikely and adds weight to the conclusion drawn above that the fluorescence kinetics of chicken pepsinogen is not to be explained by inter-tryptophan energy transfer.

In view of the above we are led to the conclusion that the kinetics of the decay of fluorescence of chicken pepsinogen is a reflection of relaxation processes which one or more excited tryptophan residues in the protein undergo following electronic excitation. In this connection it is pertinent to note the study of Brand and Gohlke (1971) who have shown that 2-*p*-toluidinylnaphthalene-6-sulfonate adsorbed on bovine serum albumin exhibits nanosecond time resolved fluorescence spectra. Similarly, the dynamic quenching of tryptophan fluorescence in proteins by molecules of oxygen led Lakowicz and Weber (1973) to the conclusion that the diffusion of the oxygen throughout the protein molecules is very facile and that the protein molecules undergo very fast relaxations to permit this to occur. The relaxation processes observed in the present study for excited tryptophan residues may be due to interaction with the environment due to reorientation of surrounding groups or formation of exciplexes (Longworth and Battista, 1970). Such reactions are usually too fast to be observed in fluid media by fluorescence techniques unless the fluorescence decay time is dramatically shortened and shifted to the picosecond time scale, as was recently done by quenching with oxygen under high pressure (Weber and Lakowicz, 1973). However, relaxation reactions in the nanosecond time scale were shown to take place in the case of diketopiperazines of tryptophan (Donzel *et al.*, 1974). The analogy between the behavior of tryptophan residues inside a protein and those in low molecular weight compounds cannot be drawn of course too closely and the relaxation processes in the two cases probably involve different reactions; in the case of proteins the relatively slow relaxation processes may result from the compact structure and resulting steric hindrances inside the protein molecule.

In conclusion it may be noted that the relaxation processes found for the tryptophan residues in pepsinogen are not necessarily confined to these residues. The experimental circumstances are such that the indole side chains are readily perturbed by electronic excitation and that the relaxation of the excited chromophore can be followed experimentally by study of the fluorescence decay of the chromophore. The findings described above for tryptophan residues may thus have wider implications, pointing to the possibility of the occurrence of nanosecond relaxation processes in other side chains as well as in proteins.

Acknowledgments

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A Multiple Wavelength Analysis of the Reaction between Hydrogen Peroxide and Metmyoglobin†

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ABSTRACT: The spectra of reacting solutions of hydrogen peroxide and metmyoglobin may be accounted for by a Beer's law combination of the molar absorptivities times the concentrations of three components: one reactant, metmyoglobin; and two products, a red pigment ferrimyoglobin peroxide (Mb IV of George and Irvine, 1955) produced in alkali, and a green pigment (MMb_{586} of King and Winfield, 1966). The kinetics of the reaction have been studied over a range of pH; 3 mol of peroxide was required at acid pH, 2 mol in alkali. The reaction proceeds through the formation of a red intermediate and some ten different reactions are involved. The formation of the green pigment is the result of the oxidation of a histidine residue as shown by a kinetic analysis of the reaction and by titration studies.

When Kobert (1900) first observed that hydrogen peroxide reacted with methemoglobin to form a red pigment he noted the formation of three diffuse bands from 500 to 513, 545 to 558, and 584 to 600 nm. Keilin and Hartree (1935) later concluded that the 500–513-nm band was due to unreacted methemoglobin, and ascribed the other two to ferrihemoglobin peroxide. This interpretation stood until King and Winfield (1966) demonstrated that the reaction of hydrogen peroxide with metmyoglobin produced at acid pH values a separate pigment form with only one absorption band at 586 nm. George and Irvine (1952) studied metmyoglobin at alkaline pH values and observed that the red pigment ferrimyoglobin peroxide[1] (PMetMb)[2] was produced without spectral variations in the pH range 8.0–9.0. The spectrum of the alkaline pigment had the same three bands as observed by Kobert with an absorption maximum at 547 nm, a low peak at 580–590 nm, and a low shoulder at 510–520 nm. Under the conditions that George and Irvine studied the pigment, it is unlikely that their pigment was contaminated with either unreacted MetMb or the green pigment. Finally, King and Winfield (1966) described what appeared to be a reaction intermediate which absorbed at 525 nm.

The conclusion from these observations is that if the acid reaction produces a green pigment and the alkaline reaction produces the PMetMb, reactions at intermediate pH values must produce varying mixtures of the two pigments. Although studies have been carried out at various intermediate pH values (George and Irvine, 1952; King and Winfield, 1963; King *et al.,* 1967; Brill and Sandberg, 1968; Yonetani and Schleyer, 1967), no systematic study has been made of the effect of pH on the production of the two pigments. In some studies, conclusions have been drawn concerning "a reaction product" where not one but two products must have actually been produced. In addition to the spectral changes that take place during the reaction, George and Irvine (1955) observed that about 0.8 mol of H^+ was released/mol of MetMb reacted with H_2O_2 at pH 8. We undertook spectrophotometric and titrimetric studies of the effects of pH and peroxide concentration in order to determine the relative amounts of pigments formed and to derive information on the mechanism of the reaction from its kinetics. In order to do so, we first had to establish whether or not the spectra obtained during the course and at the end of the reaction were produced by a Beer's law combination of the absorption spectra of identifiable compounds, and how many such compounds are produced during the reaction.

Experimental Procedures

The preparation of myoglobin has been described elsewhere (Nicholas and Fox, 1969). Pigment concentration

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[1] According to George and Irvine's (1955) interpretation this compound is but a special case of a class of compounds with iron in the ferryl (Fe^{4+}) oxidation state, to wit, ferrylmyoglobin. In this paper we will use the term ferrimyoglobin peroxide since we are concerned with only hydrogen peroxide derivatives. We will use the term acid ferrimyoglobin peroxide for the green pigment produced at low pH values. Previous usage has merely identified the pigment as the "586 complex" (King and Winfield, 1966) but identifying pigments by their absorption maxima is not too satisfactory, if for no other reason than that our spectrophotometer records the maximum at 589 nm.

[2] Abbreviations used are: MetMb, metmyoglobin; PMetMb, ferrimyoglobin peroxide; HPMetMb, acid ferrimyoglobin peroxide; RMetMb, a red intermediate formed during the reaction; MetMbOH, alkaline metmyoglobin.