FLUORESCENCE DECAY OF LYSOZYME AND OF IODINE OXIDIZED LYSOZYME

Giora Y. YASHINSKY*
The Weizmann Institute of Science, Rehovot, Israel

Received 13 July 1972

Introduction

Fluorescence spectroscopy is a useful tool in the study of conformational changes, polarity of binding sites, and energy transfer in proteins [1–4]. Due to the complexity of the systems studied, the interpretation of the results obtained by this technique often resorts to simplifying assumptions. It is therefore desirable to extend, whenever possible, the information to be obtained from fluorescence measurements. Since the quantum yields of a fluorescent species is proportional to the integral of the fluorescence decay curve with respect to time, one might expect more detailed information from fluorescence decay than from quantum yield data. The importance of such detailed information is exemplified in the present communication dealing with the fluorescence of the tryptophan residues of hen egg white lysozyme and of the iodine oxidized enzyme.

Hen egg white lysozyme contains six tryptophan residues per molecule. The indole side chain of tryptophan 108 (Trp-108), can be specifically oxidized by iodine to indole, which is essentially non-fluorescent, and which possesses distinct spectral properties. Teichberg and Sharon [5] have recently investigated the fluorescence spectra and quantum yields of lysozyme and of the iodine oxidized enzyme. Assuming that the emission characteristics of the unmodified tryptophan residues of the oxidized lysozyme and those of the corresponding residues of the native enzyme are the same, the above authors concluded that the contribution of Trp-108 to the fluorescence yield of native lysozyme amounts to 56%. The experimentally determined fluorescence decay data obtained for lysozyme and oxidized lysozyme, to be discussed below, suggest that the above assumption is an oversimplification and that the emission of the different tryptophan residues are most likely affected as a result of the oxidation of Trp-108.

2. Materials and methods

Hen egg white lysozyme (EC 3.2.1.17; Worthington, LY 8HB 8800 U/mg) was used as purchased. Iodine oxidized lysozyme, in which the indole chromophore of Trp-108 has been selectively oxidized to oxindole, was prepared by V.I. Teichberg according to Hartdegen and Rupley [6].

All fluorescence measurements were carried out using aqueous solutions of lysozyme or modified lysozyme in 0.1 M phosphate buffer, pH 8.0, at room temp. (~ 23°). Fluorescence spectra and quantum yields were obtained using a Turner Model 210 spectrofluorometer. The optical density of the solutions used at the wavelength of excitation (280 or 295 nm) was in the range of 0.1–0.05. Fluorescence decay curves were obtained using a nanosecond decay fluorometer similar to the one described by Berlman [7]. The excitation light pulse was generated by a TRW deuterium light source and monochromated by a Jarrel-Ash 0.25 m monochromator. The excitation wavelength used was 280 nm. The emitted light was

* Deceased July 8th, 1971. This communication is part of an M.Sc. thesis submitted by Giora Y. Yashinsky to the Feinberg Graduate School of the Weizmann Institute of Science and supervised by I.Z. Steinberg and E. Katchalski. Part of the work was carried out together with G. Hazan and A. Grinvald, who together with E. Haas were helpful in the preparation of the material for print. Inquiries regarding this communication should be addressed to I.Z. Steinberg or E. Katchalski, The Weizmann Institute of Science, Rehovot, Israel.

passed through a Corning (0-54) filter and detected by a RCA 1P28 photomultiplier. The electric signals obtained were monitored by a Tektronix 1S1 sampling unit and averaged on a Hewlett-Packard Model 5401 A multichannel analyser. Special precautions were taken to minimize non-linear and drift effects. A detailed description of the instrument and its mode of operation will be given elsewhere.

The fluorescence decay functions, $i(t)$ of the substances studied were obtained by a least squares analysis using the convolution equation

$$F(t) = \int_0^t G(t-s)i(s)\,ds, \qquad (1)$$

where $G(t)$ is the excitation flash profile, as detected by the experimental set-up, and $F(t)$ is the response of the detection system to the fluorescence induced by the excitation pulse. The function $i(t)$ was assumed to be a sum of exponential terms $\alpha_i \exp(-t/\tau_i)$. The parameters α_i and τ_i yielding the least square deviations between the computed and experimental curve were obtained by the NLIN computer programme [8, 9] for least squares estimation of non-linear parameters.

3. Results and discussions

The profiles of the excitation flash lamp (curve A) and of the induced fluorescence of lysozyme (curve B) as detected by the nano-second decay fluorometer, are given in fig. 1. Curve A was convoluted with a single exponential decay or with a sum of two exponential decays, and the resulting convolutions were compared with curve B by the least-squares criterion. The parameters for the decay curves which yielded the best fit are summarized in table 1. Assuming a mono-exponential decay for lysozyme, a minimal standard deviation of about 9×10^{-3} is obtained**. If the decay is assumed to be bi-exponential the standard deviation drops markedly to yield a value of less than 2×10^{-3}. The fitting of the data to a three exponential decay did not seem worthwhile because of the noise level of the measurements. The latter was estimated by analysis of the decay curve of a fluorescent substance which is expected to be mono-exponential. As seen in table 1, the fluorescence decay response of *N*-acetyl-L-tryptophanamide might be fitted to a single exponential decay with a standard deviation of about 2.8×10^{-3}.

A decay time of 1.9 nsec for the fluorescence of lysozyme was obtained by Chen et al. [10] assuming a mono-exponential decay for the enzyme. This decay time if in fair agreement with the results obtained here if a similar assumption is used. However, the data presented in the last column of table 1 shows that the fluorescence decay of lysozyme is definitely not mono-exponential. It can be fitted best to the sum of two exponential decays possessing lifetimes of 2.6 and 0.9 nsec. The lysozyme fluorescence may actually be composed of more than two exponential decays; this possibility cannot be evaluated, however, at the noise level attainable by present measurements.

The finding that the fluorescence decay of lysozyme is not mono-exponential leads to some interesting conclusions. The various tryptophan residues of the protein are obviously not luminous to an equal extent. Furthermore, the occurrence of energy exchange, if at all, among the tryptophan residues of lysozyme is not complete within the fluorescence lifetime, since a mono-exponential decay would be expected for such a case. This conclusion is in accord with that of Elkana, which was arrived at from different experimental data [11].

The response of the nanosecond fluorometer to the emission of iodine-oxidized lysozyme is also included in fig. 1 (curve C). The estimated parameters describing the decay of the fluorescence of oxidized lysozyme given in table 1 show that the decay can be described by a sum of two exponents with lifetimes of 3.0 and 0.8 nsec. Again, the possibility that the fluorescence decay is composed of more than two exponential decays cannot be ruled out.

A simple relationship exists between the fluorescence lifetime, τ, of a chromophore and its quantum yield η. Thus,

$$\tau = \tau_0 \eta \qquad (2)$$

where τ_0 is a constant equal to the reciprocal of the rate constant of decay due to radiation [12]. Among chromophores of the same kind, long lifetime is thus

** The units of light intensity for $F(t)$ were chosen in such a way that $F(t)$ assumed a value of unity at its maximum.

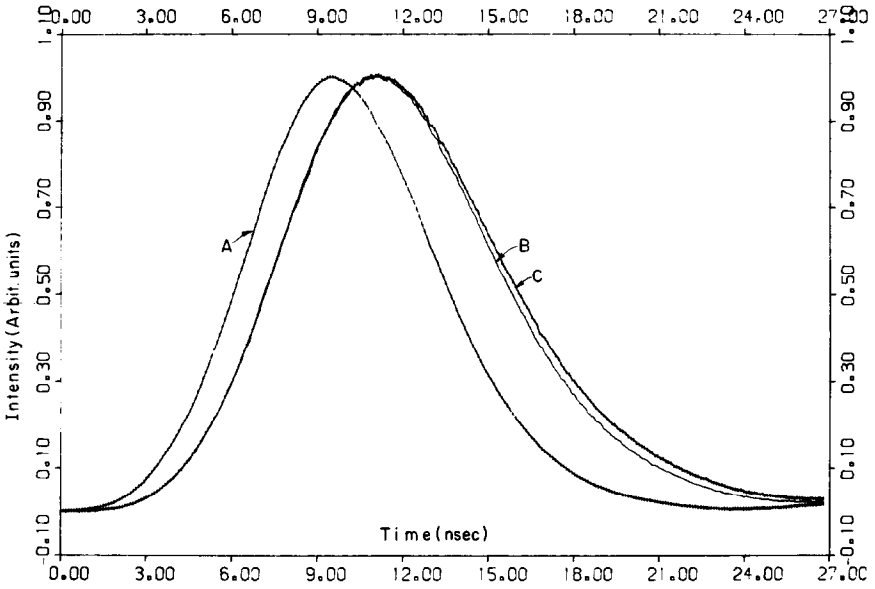

Fig. 1. Intensity profiles as detected by the nanosecond decay fluorometer. Curve A) Excitation flash; curve B) fluorescence of lysozyme in response to the excitation flash; curve C) fluorescence of iodine-oxidized lysozyme in response to the excitation flash.

Table 1
Least squares estimates for the parameters for the fluorescence decay of lysozyme, iodine-oxidized lysozyme and *N*-acetyl-L-tryptophanamide.

	No. of assumed exponential terms	τ_1 (nsec)	τ_2 (nsec)	α_2/α_1 [c]	Root mean square deviation
Lysozyme[a]	one	1.8 ± 0.05	–	–	8.75×10^{-3}
	two	2.6 ± 0.2	0.9 ± 0.2	2.0	1.82×10^{-3}
Iodine-oxidized-lysozyme[a]	one	2.0 ± 0.03	–	–	13.4×10^{-3}
	two	3.0 ± 0.1	0.8 ± 0.1	2.3	1.77×10^{-3}
N-acetyl-L-tryptophanamide[b]	one	2.9 ± 0.02	–	–	2.79×10^{-3}

[a] Average of four experiments; the ± figures show extreme values obtained in individual experiments.
[b] Average of two experiments.
[c] Due to the relatively high correlation among parameters, the values of α_2/α_1 have only semi-quantitative significance.

associated with high quantum yield and vice versa. The data obtained for lysozyme thus show that the tryptophan residues in lysozyme are not equally luminescent; one or more of the tryptophan residues in this enzyme have a relatively high quantum yield whereas other tryptophan residues have a relatively low quantum yield.

Teichberg and Sharon [5] have recently reported that the fluorescence quantum yield of iodine-oxidized lysozyme amounts to 44% of that of native lysozyme. A similar value was obtained for the quantum yield of iodine-oxidized lysozyme relative to the native enzyme (40%) in this study. Taking into consideration the specific oxidation of Trp-108 by iodine [6] and tacitly assuming that upon oxidation of Trp-108 no change in fluorescence properties of all the other tryptophan residues of the protein occurs, Teichberg and Sharon concluded that Trp-108 of lysozyme contributes 56% to the fluorescence

of the native enzyme. Such a conclusion would demand that the exponent with the long lifetime of lysozyme is due to Trp-108 while the exponent possessing the short lifetime is due to some of the other tryptophan residues in the molecule, each of them having a relatively low quantum yield. However, if one adheres to the above asssumption, the long-lived exponent of lysozyme which has been attributed to Trp-108, should not appear in oxidized lysozyme. The data presented in table 1 clearly show that this is not the case. Oxidized lysozyme does exhibit an exponent in its fluorescence decay possessing a long lifetime, which in fact is somewhat longer than the corresponding one in lysozyme. No proof seems, therefore, to be available that Trp-108 contributes most of the fluorescence of lysozyme.

The drop in fluorescence intensity accompanying the destruction of Trp-108 must therefore be due, at least in part, to the diminution of the fluorescence intensity of some tryptophan residues other than Trp-108. A detailed discussion of the correlation of the fluorescence decay data reported here with the corresponding quantum yields will be published elsewhere. The changes in the fluorescence of the tryptophan residues other than Trp-108 upon conversion of lysozyme into oxidized lysozyme are probably due to concomitant changes in protein conformation. In this connection it is pertinent to note that differences between the conformations of lysozyme and iodine-oxidized lysozyme have indeed been detected by X-ray diffraction techniques [13, 14].

References

[1] G.M. Edelman and W.O. McClure, Accounts Chem. Res. 1 (1968) 65.
[2] L. Stryer, Science 162 (1968) 526.
[3] L. Stryer, in: Ciba Found. Symp., eds. R. Porter and M. O'Connor (Churchill, 1970) p. 133.
[4] I.Z. Steinberg, Ann. Rev. Biochem. 40 (1971) 83.
[5] V.I. Teichberg and N. Sharon, FEBS Letters 7 (1970) 171.
[6] F.J. Hartdegen and J.A. Rupley, Biochim. Biophys. Acta 92 (1964) 615.
[7] I.B. Berlman, Handbook of Fluorescence Spectra of Aromatic Molecules (Academic Press, 1965).
[8] D.W. Marquardt, NLIN, submitted by Share Programme Library, SDA, 3094 (1966).
[9] P.C. Lichtenberg, J.R. Stevens and T.D. Newton, Can. J. Phys. 50 (1972) 345.
[10] R.F. Chen, G.G. Wurek and N. Alexander, Science 156 (1967) 948.
[11] Y. Elkana, J. Phys. Chem. 72 (1968) 3655.
[12] J.B. Birks and I.H. Munro, in: Progress in Reaction Kinetics, Vol. 4, ed. G. Porter F.R.S. (Pergamon Press, 1967) p. 289.
J.B. Birks, in: Photophysics of Aromatic Molecules (Wiley-Interscience, London, 1970) pp. 100–108.
[13] C.R. Beddel and C.C.F. Blake, in: Chemical Reactivity and Biological Role of Functional Groups in Enzymes, Vol. 31, Biochem. Soc. Symp. ed. R.M.S. Smellie (1970) p. 157.
[14] C.R. Beddel, Ph.D. thesis, Oxford University (1971).