VACUUM ULTRAVIOLET STUDIES ON THE NATURE OF THE RADIATION INACTIVATION OF TRYPSIN

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ABSTRACT Trypsin, in powder form and in frozen D_2O-glucose solutions, at temperatures from 100° to 300°K, was excited with vacuum ultraviolet and near ultraviolet radiation to determine how absorbed photon energy is partitioned into radiative, nonradiative and/or inactivating processes; at 300°K most of the absorbed energy is not reemitted, so that it (0.98–0.99 for excitation at 120 nm and 0.75–0.90 at 280 nm) is potentially available for inactivation. Since the effects of excitation wavelength and temperature on the emission quenching yields are generally different from those on the inactivation yields of dry trypsin, the mere retention of quenched energy by an enzyme does not necessarily lead to its inactivation. Thus, as predicted previously, the radiation inactivation of trypsin must proceed by rather specific mechanisms which undoubtedly depend upon environment-sensitive processes, since the nature of the molecular environment can modify the partitioning of energy so significantly; for example, there are differences in the phosphorescence-to-fluorescence ratio, in the activation energy for quenching, and in the lifetimes and kinetics of the decay of phosphorescence when trypsin in frozen glasses and dry trypsin are excited by various wavelengths of ultraviolet radiation.

INTRODUCTION

There has been a question for a number of years as to whether there are common mechanisms underlying enzyme inactivation by the ionizing radiations and by light in the near ultraviolet (NUV) region of the spectrum (e.g., reference 1). Evaluation of the various possibilities is complicated by the fact that whereas NUV photons can excite molecules only to the lowest-lying excited levels, the ionizing particles can both ionize and also cause excitation directly to higher-lying excited states (2, 3). Since it is not possible to produce ionizations alone, the most promising way to separate the contributions of these two types of processes is to look at the effects of excitations to the higher-lying states. We have attempted to do this by investigating the emissive properties of the enzyme trypsin—which has been studied exten-

sively in radiation biology—following excitation by photons in the vacuum ultraviolet (VUV) portion of the spectrum.

As a means of further evaluating possible enzyme inactivation mechanisms, we have directed our attention to some physical factors which influence the way in which absorbed energy is partitioned within an enzyme. In particular, we have been concerned with how temperature, exciting wavelength, and/or the environment surrounding an enzyme molecule determine (*a*) how much energy is retained within the singlet manifold and reemitted as fluorescence; (*b*) how much intersystem crossing occurs to the triplet manifold with subsequent phosphorescence emission; and (*c*) how much energy is quenched and thus retained by the molecule for potential chemical changes which might lead to loss of enzymic function. Portions of this research were also designed to investigate further the similarities and differences between NUV- and X-ray–induced emission described earlier (4, 5). Similar studies on the aromatic amino acids are reported elsewhere.[1]

MATERIALS AND METHODS

The details of the control of sample temperature, the specifications of the NUV-VUV McPherson Instrument Company (Acton, Mass.) spectroscopic equipment, the collection and treatment of the data, and an evaluation of the sources of error are contained in a paper to be published.[1] The trypsin used was obtained from Worthington Biochemical Corporation (Freehold, N.J.) as a twice crystallized, salt-free, lyophilized preparation and was sifted through a 100-mesh sieve as the first step in preparing the powdered samples for study. This powder was mixed with a minimal amount of methanol to form a paste for application onto the metal cold-finger. After drying, the resultant film for irradiation was about 0.1 mm thick. For studies in solution the trypsin was dissolved in D_2O containing 0.5% glucose; at 77°K this forms a glass which is transparent to wavelengths above about 170 nm (6, 7).

RESULTS AND DISCUSSION

Comparison of Enzyme Inactivation and Emission Yields

Fig. 1 gives the excitation spectra for fluorescence and phosphorescence[2] from trypsin powder at 100°K. The ordinate gives the emission quantum yields relative to that of solid sodium salicylate at 300°K. Clearly the emission yield of trypsin powder decreases by a factor of about 10 when the excitation energy is changed from NUV

[1] Yeargers and Augenstein. Paper to be published.

[2] In reporting our present results, we use P/F to designate the emission occurring with life-times long enough ($>$ 10 msec) to pass through our rotating shutter divided by that with shorter life-times in the interval 285–350 nm. The reason for this is that there was not sufficient intensity to allow us to detect phosphorescence after it had passed through our monochromator. Thus, our "*P*" may contain emissions of higher multiplicity (5) and our "*F*" could include some delayed fluorescence.

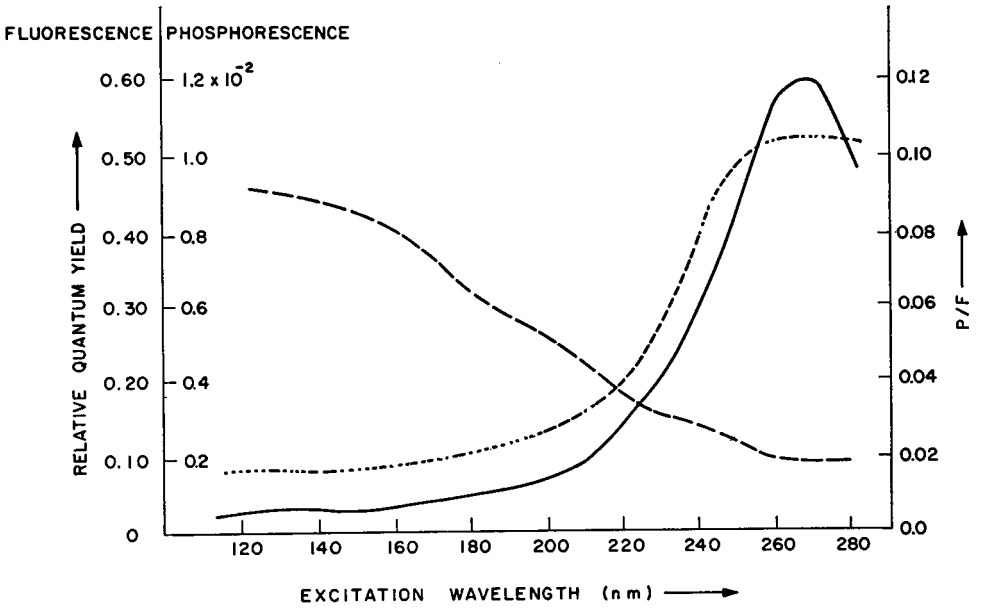

FIGURE 1 The phosphorescence (dotted line) and fluorescence (solid line) excitation spectra and the P/F (dashed line) values for trypsin in dried powders at 100°K. The yield ordinates are given relative to the fluorescence yield of dry sodium salicylate at 300°K.

to VUV. Although these relative values are probably accurate to ±20%,[1] the absolute values unfortunately cannot be given with comparable certainty; i.e., there are several published values for the emission yield, Y_{es}, of sodium salicylate, which vary from 0.25 to 0.98 (see reference 8).

Setlow has reported the inactivation quantum yield for solid trypsin irradiated by NUV and VUV light at 300°K (9). In Fig. 2 his data are reproduced for comparison with our estimates of the emission quenching yield, Y_{qt}, for trypsin at 300°K[3]; the two curves shown for quenching are based on the two extreme estimates for Y_{es}.

Y_{qt} represents the fraction of photons absorbed which *do not* result in light emission, and is given by $Y_{qt} = 1 - Y_{et}$, where Y_{et} is the absolute emission quantum yield for trypsin. Thus, it seems worth while to compare Y_{qt} with inactivation yields, since quenching is a necessary prelude to inactivation. Obviously, neither of the curves for Y_{qt} is similar in shape to the inactivation plot of Setlow.[4] This implies immediately that inactivation does not necessarily follow with some constant probability the quenching of a fixed amount of energy. This is consistent with earlier conclusions based on studies with X-ray excitation (4).

[3] The shape of the excitation spectrum for emission at 300°K is the same as that for fluorescence at 100°K, but the emission yield is reduced to about 50% of the latter value.

[4] Because of the small emission yields, the 20% error in such measurements becomes only a 1–2% error in our quenching curves.

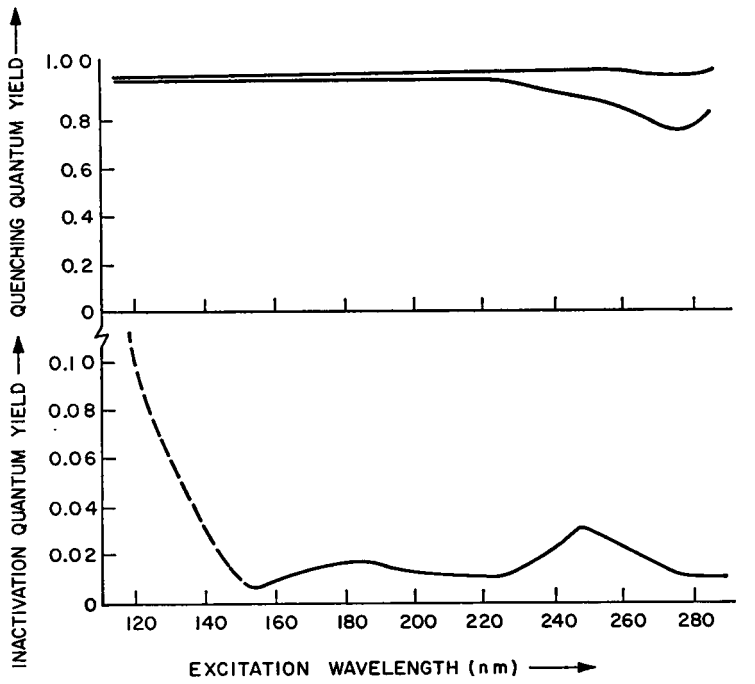

FIGURE 2 *Top:* The quenching spectra for powdered trypsin at 100°K. The values of Y_{qt} on the ordinate give the fraction of absorption events which do not lead to light emission at 300°K. The two curves were plotted using the extreme values reported for the emission quantum yield of sodium salicylate: 0.25 (lower curve) and 0.98 (uppermost curve) (8). *Bottom:* The quantum yield for the inactivation of dried trypsin samples at 300°K as a function of the wavelength of the exciting light (data from Setlow (9)) (Note the different ordinates in the two plots.)

This conclusion is further buttressed by the following considerations. Even when the uncertainty in Y_{es} is taken into account, it is clear that Y_{qt} for excitation at 120 nm is about 0.98–0.99 and is between 0.75 and 0.90 at 280 nm. When these values are converted to energy units, we obtain a value of about 0.7–0.99 as the fraction of the energy absorbed by solid trypsin in the NUV and VUV regions which is potentially available for enzyme inactivation (i.e., *not reemitted*); as a specific example, if 500 ev of 160-nm radiation is absorbed by a trypsin crystal at 300°K, less than 30 ev will be reemitted. However, Setlow reports that an average of only one molecule of trypsin will be inactivated by the absorption of these 65 quanta, even though the UV inactivation of trypsin follows "one-hit" kinetics (10, 11). Thus, inactivation depends upon a single event involving either only one specific mechanism of many initiated by absorption, or else one which produces inactivation with very low probability.

If we make the reasonable assumption that the absorption spectra of chymotrypsin and trypsin are similar (see reference 9 and references therein), the present data

can be used to calculate the average amount of energy absorbed by dry trypsin per quantum emitted (i.e., the inverse of yield). Absorption by chymotrypsin increases rapidly between 240 and 200 nm and is approximately constant below that; thus, if the excitations caused by the electrons produced by X-rays obey optical selection rules, the major portion of the energy losses should be greater than about 6 ev. Using this approximation and the data in Fig. 1, we calculate that for a uniform spectrum of subionizing photons (<10 ev) trypsin should absorb about 300–500 ev per quantum emitted. This is larger than the value of 124 ev absorbed from X-ray excitation per quantum emitted (12). This difference presumably reflects the effects of ionization, and, in particular, indicates that X-irradiation generates slow electrons which do not produce excitations in accordance with optical selection rules (5, 13).

The Dependence of Emission on Temperature

The sample temperature at time of irradiation has been shown to be one of the factors which most influence enzyme inactivation yields: with ionizing particles the yields at 400°K may be 5 times as great as those at 77°K for some enzymes. Further, the temperature dependence at high and low temperatures is quite different; the thermal coefficients associated with inactivation correspond to activation energies of essentially zero for $T < 100$°K and 0.1 ev/molecule for $170° < T < 400$°K (14). For dry trypsin, increasing the temperature at which NUV irradiation is carried out from 90°K to 300°K increases the inactivation quantum yield by a factor of 3 (11).

In order to investigate the temperature dependence of emission quenching (and so compare it with that for inactivation), we have plotted our results on the basis of the assumption that the data can be described by

$$Y_{qt} = \exp(-E_{qt}/kT)$$

where E_{qt} is the thermal activation energy for emission quenching. Fig. 3 is such a Boltzmann plot of log Y_{qt} and also of log Y_{et} vs. $1/T$ for fluorescence of solid trypsin and D_2O-glucose glasses containing trypsin. The data for phosphorescence are not shown, since Y_{qt} for phorphorescence, even at low temperatures, is so large that the quenching activation energy will always be very small (<0.001 ev). Since the present values for activation energies do not agree with previous values for either the X-ray–induced fluorescence or phosphorescence from trypsin (4), the mechanisms initiated by the two types of radiation must not be identical.

The present data demonstrate that the emission quenching yield has only a weak dependence on temperature. Thus, there must be additional, temperature-dependent steps between emission quenching and inactivation. This conclusion is consistent with our earlier observation that it is not the general presence of quenched energy per se which leads to inactivation, but rather the presence of sufficient energy at a specific site in the protein; the argument that enzyme inactivation depends upon

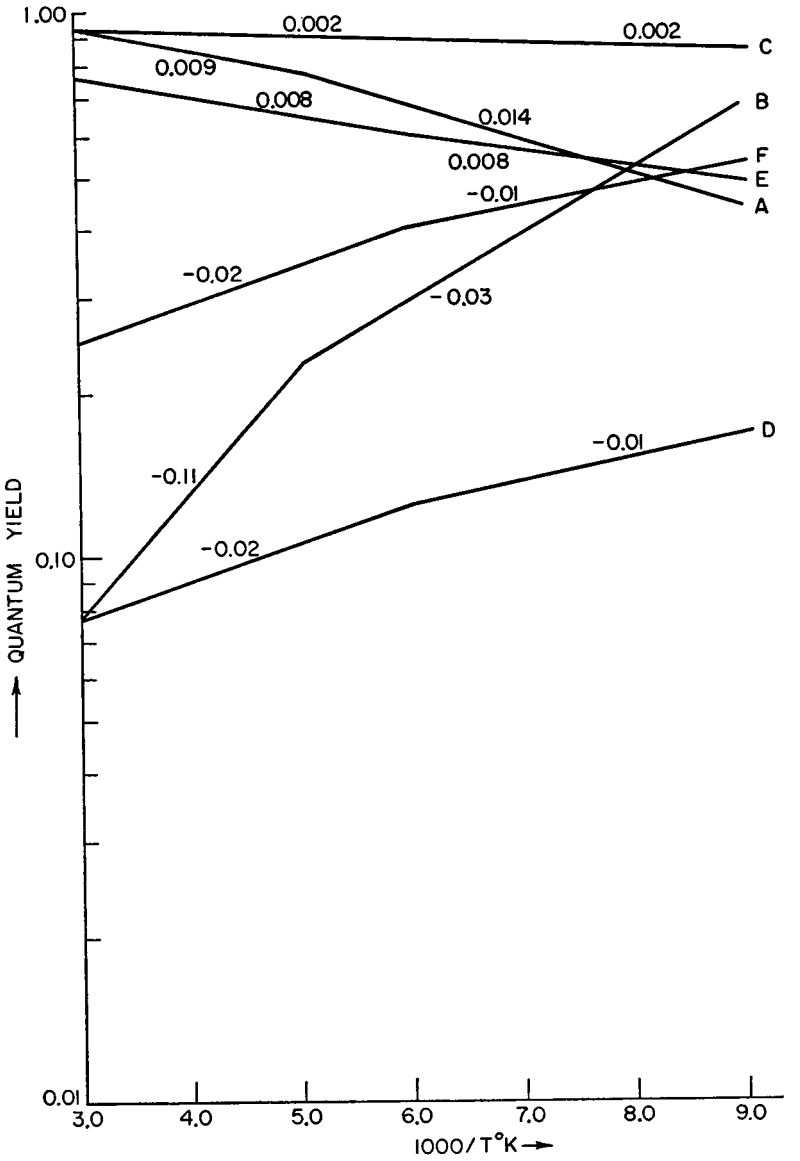

FIGURE 3 The dependence of various emission yields on the reciprocal of temperature. A, the quenching of fluorescence for trypsin dissolved in a D_2O-glucose solution; B, the emission data corresponding to curve A, normalized at 300°K for the yield given by Teale (24); C and E, the quenching of the fluorescence from trypsin powders based on the two extreme estimates of the emission yield of dry sodium salicylate given in the literature (see text and legend of Fig. 2); and D and F, the emission data corresponding to curves C and E, respectively. Activation energies are given in electron volts per molecule.

All phosphorescence quenching activation energies are very small (< 0.001 ev/molecule) owing to the low phosphorescence emission yields.

specific damage has been advanced previously on the basis of other studies (e.g., 15–17).

Of particular consequence is the observation that the effect of temperature on trypsin emission was the same for excitation with 280-, 220-, 160-, or 135-nm light. Accordingly, the rate-limiting process for the thermal quenching of emission must be associated with a radiationless transition between the lowest excited state and the ground state. There must be other, temperature-independent processes which contribute to quenching, however, since the excitations to higher excited states give a lower emission yield than those to the first excited state.

i) This could occur if higher excited states are quenched directly to the ground state (bypassing the first excited state) in competition with first excited state–to–ground state quenching by appropriate crossing of potential energy curves. Such a process would require that the direct quenching of upper excited states take place in about 10^{-13} to 10^{-11} seconds in order to compete with energetic relaxation processes leading to the first excited state. An energetic relaxation time of this magnitude would necessitate very strong coupling between the pertinent upper excited state and the ground state—a situation which seems unlikely in view of their large energy separation (18).

ii) Configuration changes could provide another means for bypassing the lowest-lying emitting state. That is, a molecule in a higher-lying state may be very susceptible to bond rearrangements to give a molecule with altered spectroscopic properties.

iii) Alternatively, a molecule, having relaxed to the lowest excited level from a higher state, may "remember" its history sufficiently to affect its subsequent probability of emitting radiation. Such a "memory" device could arise from the environmental perturbations (e.g., lattice distortion) due to the energy dissipated into vibrations during the relaxation from the higher states.

The Influence of the Molecular Environment

The present results indicate that the specific processes mentioned above depend critically upon the nature of the surroundings of the molecule. Previous results have also shown that the molecular environment determines, to a large extent, how excitation energy is partitioned among the various processes of emission, dissipation, and/or inactivation. For example, the ability of certain residues in a protein to transfer energy or undergo intersystem crossing was found to be a function of their environments and the exciting wavelength (19, 20). Specifically, the phosphorescence/fluorescence (P/F) ratios and the fraction of phosphorescence from tryptophan and tyrosine residues varied from protein to protein, and changed when 240-nm UV was used for excitation rather than 280-nm (21). Further, the phosphorescence from tyrosine at 77°K is different for this amino acid in frozen solution, in lyophilized powders, in homopolypeptides, and in the protein ribonuclease (22).

Two portions of the present data further confirm the important role of molecular environment in determining the partitioning of excitation energy. (*a*) The *P/F* ratio from trypsin powders increases by almost a factor of 5 as the exciting wavelength is changed from 280 to 120 nm: *F* decreases by about 25 and *P* by less than 6 (Fig. 1). In the D_2O-glucose preparations, however, both *F* and *P* change by about the same factor of 10, so that the *P/F* changes very little for excitation in the interval 160–280 nm (Fig. 4). Thus, not only is there extensive quenching following excitation to the higher-lying excited states, but in the solid preparations some process(es) enhances

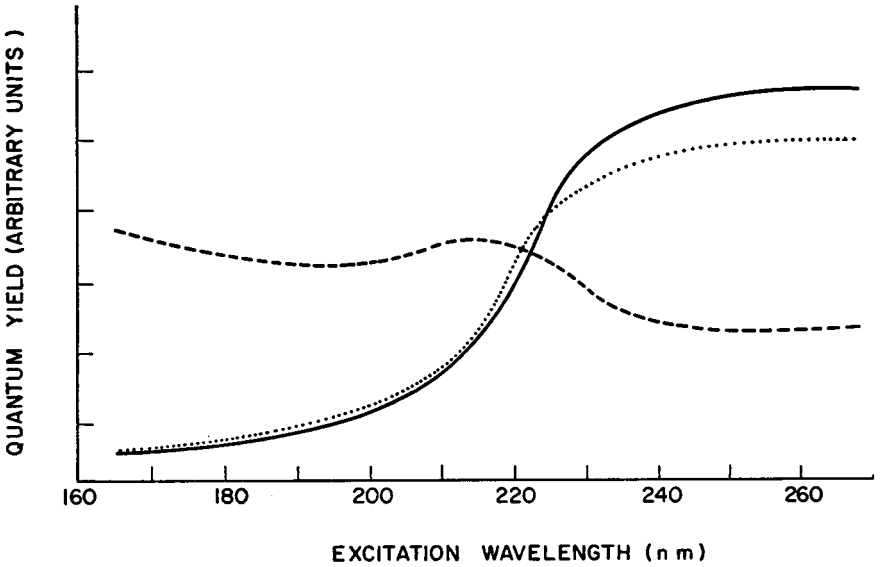

FIGURE 4 The phosphorescence (dotted line) and fluorescence (solid line) excitation spectra and the *P/F* (dashed line) values for trypsin dissolved in a D_2O–0.5% glucose glass maintained at 100°K. The *F* and *P* emission yields were determined only on a relative basis. Previous determinations of the *P/F* for trypsin in frozen solvents have varied from 0.15 to 0.22 (5, 19).

intersystem crossing during this vibrational relaxation; the constancy of *P/F* in the D_2O-glucose preparations indicates that a similar enhancement of intersystem crossing did not occur in the frozen solutions at neutral pH (see reference 23). (*b*) In addition, the mean lifetimes ($t_{37} \pm 0.2$ sec) of phosphorescence from trypsin powders which we measured at 100°K vary with exciting wavelengths: for excitation at 135 nm, 1.9 sec; 160 nm, 2.0 sec; 220 nm, 2.6 sec; 280 nm, 3.6 sec. Further, the phosphorescence decay plots could all be resolved into two exponential components with t_{37}'s of about 1 sec and 4 sec, the two components being present in different proportions depending on the excitation wavelength.

This last observation could reflect the bond rearrangements mentioned earlier; i.e., different molecular species may have different emission lifetimes. However,

it seems more likely that it may be the result of emission from molecules in two different environments within the crystals, since the phosphorescence from trypsin dissolved in clear glasses, irrespective of the exciting wavelength, has a single exponential component with a $t_{37} \simeq 6$ sec.

The trend toward shorter phosphorescence decay times at shorter excitation wavelengths in the powdered samples is opposite to that observed in the three aromatic amino acids powders.[1] Further, the mean values cited above "bridge the gap" between the values of 4 sec and 2.2 sec reported previously for NUV- and X-ray–induced phosphorescence. These observations are of particular interest since X-ray–induced phosphorescence from trypsin resembles that from tyrosine in decay time, peak wavelength, and thermal coefficients (4); whereas NUV excitation leads to phosphorescence which appears to originate from the constituent tryptophan residues (19, 21, 22). It is hoped that more sensitive equipment now being constructed will allow us to disperse the VUV-initiated phosphorescence so as to determine whether the difference in spectroscopic behavior between NUV- and X-ray–excited trypsin reflects primarily the effects of excitations to higher-lying states, ionizations, or slow electrons produced by the latter radiations (5).

These various results indicate that NUV and VUV destruction of trypsin activity probably proceeds by rather specific steps which depend greatly on the nature of the macromolecular environment. Unfortunately, the extent to which this specificity reflects an intrinsic property of individual residues and their immediate environment or of internal protein organization which enhances highly specific energy transfer cannot be determined quantitatively from currently available results. Quite clearly, the present studies of comparative emission yields, phosphorescence decay, and the effects of temperature on emission intensity show that X-ray excitation must initiate preferentially some electronic mechanisms in trypsin which are not of importance in either NUV or VUV excitation.

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