Phosphorescence Studies of Environmental Heterogeneity for Tryptophyl Residues in Proteins*

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ABSTRACT: The phosphorescence spectra of horse liver alcohol dehydrogenase (HLAD), yeast alcohol dehydrogenase, papain, and trypsin reveal heterogeneity for the tryptophan residues. For HLAD which contains two tryptophans per monomer, two overlapping tryptophan phosphorescence spectra separated by 300 cm^{-1} are observed in a 1:1 ethylene glycol–buffer glass at 77°K. The relative proportions of the components in the tryptophan phosphorescence are dependent upon the wavelength of the exciting light indicating that the emitting species have somewhat different absorption spectra. A shift in the fluorescence spectrum of HLAD as a function of exciting wavelength indicates that the protein fluorescence spectrum is also a superposition of two differing tryptophan emissions. Phosphorescence data suggest that the two components arise from tryptophans either buried in the polarizable protein core or occupying an environment where they are exposed to the polar solvent. The heterogeneity in the tryptophan absorption and emission permits in general the selective excitation and observation of the individual tryptophan classes, and in the case of HLAD, the study of individual tryptophyl residues. Energy-transfer experiments in HLAD reveal: (1) different singlet–singlet-transfer efficiencies from the two tryptophans to the coenzyme NADH, and (2) triplet–triplet energy transfer from an acyl-protein label to only one of the tryptophans in the enzyme.

As a consequence of the tertiary structure of a protein, individual amino acid residues of the same species within the protein can be brought into different local environments. The microenvironments about these residues may differ with respect to their polarizability, polarity, and mobility. For the aromatic amino acid residues, it would be anticipated that these variations in local environment would effect the electronic transition energies which are observed in absorption and emission. It is known that the absorption and emission spectra of free tryptophan are influenced by solvent (Konev, 1967; Yanari and Bovey, 1960) and that the absorption spectrum for exposed tryptophan residues in a protein can be perturbed by changes in the dielectric constant of the medium (Herskovits and Laskowsky, 1960). The tryptophan fluorescence spectrum, quantum yield, and lifetime are observed to differ for proteins in their native but not in their denatured forms (Teale, 1960). Furthermore, a shift in the emission maximum is observed in the fluorescence spectrum of several proteins upon partial quenching of the tryptophan emission by energy transfer to a singlet acceptor (Lehrer and Fasman, 1967; Elkana, 1968). These data have been interpreted in terms of two components in the fluorescence, corresponding to tryptophans in different environments (Konev, 1967).

In the present study we have observed the fluorescence and phosphorescence from several proteins. We show that at least two classes of tryptophans in these proteins can clearly be resolved in absorption and emission spectra. These two classes appear to represent buried and exposed residues in the protein.

The observation that the tryptophans in proteins can be divided into spectral classes permits the selective study of excitation transfer and of excited-state processes in general within each class. Excitation energy transfer has been widely observed both between the aromatic residues in proteins (Weber, 1960) and between these intrinsic chromophores and prosthetic groups (Velick, 1961) or extrinsic chromophores (Stryer, 1968). This nonradioactive transfer is primarily of the long-range singlet–singlet type. The theory for this type of transfer has been developed by Förster (1948, 1966) and experimental verification for the theory has been obtained (Latt *et al.*, 1964; Stryer and Haugland, 1967; Haugland *et al.*, 1969). In addition it has been shown recently that triplet–triplet energy transfer can also be observed in proteins and can be used to probe for the proximity of tryptophans to the active site of enzymes (Galley and Stryer, 1968).

In the present article we show that within horse liver alcohol dehydrogenase (mol wt 80,000), a dimeric enzyme which contains only two tryptophans per monomer (Cannon and McKay, 1969), singlet–singlet transfer to NADH can be decomposed into a separate transfer efficiency for each of the two tryptophans. In addition, triplet–triplet energy transfer from a phenacyl group coupled to horse liver alcohol dehydrogenase is shown to occur to only one of the two tryptophans in the molecule.

Materials and Methods

Papain (twice-crystallized suspension) was obtained from Worthington and Schwarz; horse liver alcohol dehydrogenase (once crystallized and lyophilized) was purchased from Worthington, Schwarz, and Sigma. Yeast alcohol dehydrogenase (twice crystallized and lyophilized) and trypsin (twice crystallized, salt free, and lyophilized) were Worthington

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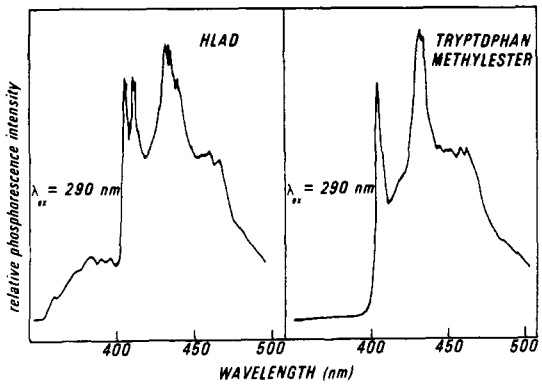

FIGURE 1: Phosphorescence spectra of 10^{-4} M HLAD and 5×10^{-4} M tryptophan methyl ester in 1:1 ethylene glycol–buffer glasses at 77°K.

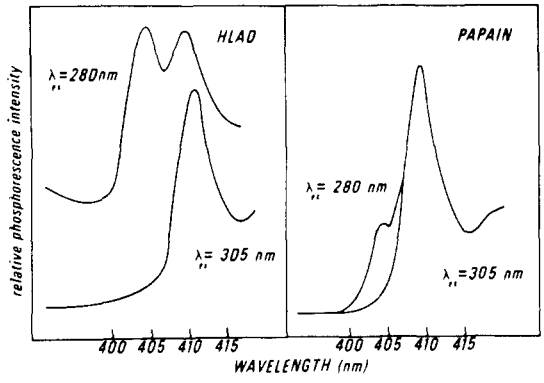

FIGURE 2: Short-wavelength region HLAD and papain phosphorescence spectra on an expanded wavelength scale, illustrating the spectral dependence on exciting wavelength. Sample concentrations are *ca.* 10^{-4} M in 1:1 ethylene glycol–buffer glasses at 77°K.

products. P-L Biochemicals supplied NADH. L-Tryptophan methyl ester was obtained from Mann Research Laboratories. Ethylene glycol, sodium bromide, isobutyramide, and guanidine hydrochloride were Fisher Certified reagents. α-Bromoacetophenone was purchased from Aldrich Chemicals. All materials were used as obtained from the supplier without further purification. Phenacylated HLAD[1] was prepared by incubating buffered (0.05 M, pH 7.5 sodium phosphate) 5×10^{-4} M HLAD with 2 equiv of α-bromoacetophenone dissolved in a minimal amount (*ca.* 2% of final solution volume) of dioxane, for 3 hr at room temperature.

Emission spectra were recorded with a conventionally designed emission spectrometer. Exciting light was provided by either a 100-W high-pressure mercury arc (PEK Labs, Inc.) or a 150-W Bausch and Lomb xenon source. Excitation wavelengths were selected with a 250-mm (6.6-nm/mm dispersion) Bausch and Lomb grating monochromator. Coupled motor-driven choppers at the entrance and exit ports of the sample compartment permit the phosphorescence to be isolated. The choppers could be removed for right-angle fluorescence measurements. Sample emission was dispersed by a 500 mm (3.3-nm/mm dispersion) Bausch and Lomb grating monochromator fitted with an EMI (6256S) photomultiplier tube. The photomultiplier output was amplified by an electrometer and recorded along the *y* axis of a Moseley 7035B X-Y recorder. The emission monochromator wavelength drum was connected to the *x* axis of this same recorder. A Moseley 17108A time base was available for phosphorescence lifetime measurements. Monochromator slit settings were typically 1.00 mm. All spectra, unless otherwise indicated, are uncorrected.

Enzyme samples were prepared in 0.05 M, pH 7.5 sodium phosphate buffer and, for phosphorescence and low-temperature fluorescence measurements, then diluted 1:1 with ethylene glycol. Sample concentrations and cell types varied according to the requirements of each experiment. Phosphorescence and low-temperature fluorescence measurements were usually performed on *ca.* 10^{-4} M (monomer basis) enzyme samples contained in 2-mm i.d. Suprasil tubes and cooled to liquid nitrogen temperature to form a glass. With care, intensity reproducibility under such conditions was ±10% or better. Room temperature fluorescence measurements typically employed 1-cm² quartz fluorescence cuvets with enzyme concentrations varying from 10^{-4} to 10^{-6} M.

NADH quenching of tryptophan emission from HLAD was observed on 5×10^{-6} M HLAD samples in room temperature fluorescence and 2×10^{-4} M HLAD (diluted 1:1 with ethylene glycol) in phosphorescence, with 1.1 equiv of NADH in either case. Ternary complex samples consisted of 1.5×10^{-6} M HLAD, 1.1. equiv of NADH, and 10^{-1} M isobutyramide for room temperature fluorescence and 2×10^{-4} M HLAD, 1.1 equiv of NADH, and 10^{-1} M isobutyramide (diluted 1:1 with ethylene glycol) in phosphorescence.

Results

Protein Phosphorescence Spectra. The phosphorescence spectrum for tryptophan methyl ester, typical of that observed for tryptophan and other indole derivatives (Freed and Salmre, 1958), is illustrated in Figure 1. A salient feature of this spectrum is the single sharp peak, presumably the 0–0 transition, at 405 nm. Phosphorescence from horse liver alcohol dehydrogenase also shown in Figure 1, is seen to be primarily due to tryptophan. The emission occurring at shorter wavelengths has a lifetime of 2.6 sec and is known from earlier observation to be due to tyrosine (Longworth, 1961). The first tryptophan peak in the spectrum of the enzyme occurs as a distinct doublet with maxima at 405 and 410 nm. The second principal phosphorescence maximum is also resolved as a doublet with maxima at 432 and 438 nm. Phosphorescence lifetimes for the 405- and 410-nm peaks in HLAD are the same as that recorded for tryptophan methyl ester, on the order of 6 sec. Hence, tryptophan phosphorescence from HLAD occurs as a superposition of two spectra which are shifted by 300 cm^{-1} relative to each other. The position of the 405-nm peak coincides with that for free tryptophan methyl ester in a 1:1 ethylene glycol–water glass.

The observation of more than one component in the tryptophan phosphorescence spectrum in proteins is not limited to HLAD. We have obtained evidence for multiple components in the tryptophan phosphorescence of papain,

[1] Abbreviation used is: HLAD, horse liver alcohol dehydrogenase.

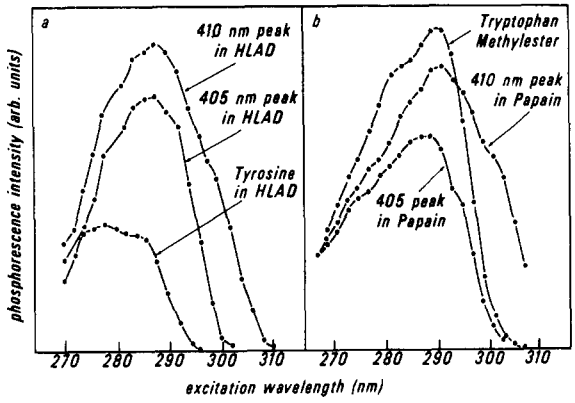

FIGURE 3: (a) Phosphorescence excitation spectra for the 405-nm and 410-nm tryptophan peaks and for tyrosine at 390 nm in HLAD. The excitation spectra are not corrected for wavelength variations in the intensity of the exciting xenon source. Enzyme concentration was 10^{-4} M in a 1:1 ethylene glycol–buffer glass at 77°K. (b) Excitation spectrum for 10^{-4} M papain observed at the 405- and 410-nm phosphorescence peaks and for 5×10^{-4} M tryptophan methyl ester in 1:1 ethylene glycol–buffer glasses at 77°K. Phosphorescence intensities among these spectra are not comparable.

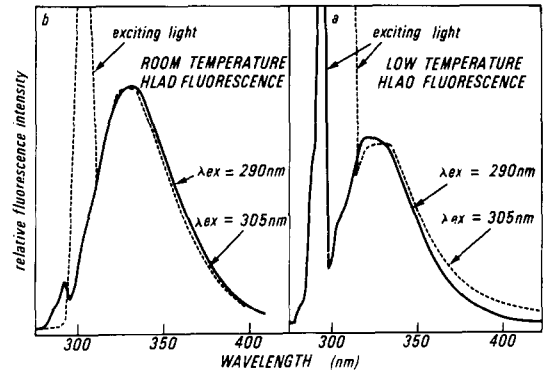

FIGURE 4: Fluorescence spectra of 5×10^{-6} M HLAD in 1:1 ethylene glycol–buffer, excited at 290 and 305 nm.

yeast alcohol dehydrogenase, and trypsin. Figure 2 displays the short-wavelength phosphorescence spectrum of papain, a molecule containing five tryptophans (Light *et al.*, 1964). In papain a single peak appears at 410 nm, but a shoulder is observed at 405 nm upon excitation with 280-nm light, suggesting that a fraction of the tryptophans in papain exhibit the higher energy emission.

Dependence of Tryptophan Phosphorescence upon Exciting Wavelength. The heterogenous nature of the tryptophan phosphorescence spectrum in proteins is more clearly established upon variation of the wavelength of the exciting light. Figure 2 shows the phosphorescence spectrum (in the 0–0 region) for papain and HLAD with excitation at the center (280 nm) and the edge (305 nm) of the protein absorption band. Clearly, 305-nm light excites only the red component of the tryptophan phosphorescence. The observation that the relative proportions of the two components of the tryptophan phosphorescence doublets are dependent on the exciting wavelength rules out the possibility that the doublets arise from the appearance of vibronic transitions not seen in the free molecule. *The heterogeneity in the spectrum can only arise from emission from independent tryptophan residues having somewhat different triplet energies.* Since only the red tryptophan phosphorescence component is observed with 305-nm excitation, the absorption spectra for the two classes of tryptophans must also be different.

Phosphorescence Excitation Spectra in HLAD and Papain. Uncorrected excitation spectra for the 405- and 410-nm phosphorescence peaks in HLAD and papain are shown in Figure 3a,b. The excitation spectrum for tyrosine phosphorescence, which begins at shorter wavelengths than that of tryptophan in the phosphorescence spectrum for HLAD, is included in Figure 3a, and that for tryptophan methyl ester appears in part b. It is apparent that the excitation spectra for the tryptophan components in the proteins are quite different. At exciting wavelengths greater than *ca.* 300 nm, the long-wavelength component in the tryptophan phosphorescence is excited exclusively. For exciting wavelengths below 300 nm both tryptophan phosphorescence components are excited. Emission from the short-wavelength component then contributes to the 410-nm emission, distorting the long-wavelength excitation spectrum below 300 nm. Contributions from tyrosine also distort the tryptophan excitation spectra at shorter wavelengths, particularly in HLAD. However, it is clear from the tyrosine excitation spectrum that both tryptophan components can be excited at longer wavelengths essentially free of tyrosine. *This difference in excitation spectra between the two tryptophan components requires that the absorption spectrum for tryptophan within these proteins must be a superposition of two absorptions differing by more than 800 cm^{-1} at the red edge.*

Fluorescence Spectra in HLAD. In view of the significant difference in the phosphorescence excitation spectra, and therefore the absorption spectra, for tryptophans within HLAD, some difference in their fluorescence spectra is to be expected. In a 1:1 ethylene glycol–buffer glass at *ca.* 200°K, the protein fluorescence spectrum excited with 305-nm light is seen (Figure 4a) to be shifted to the red of the spectrum obtained with 290-nm excitation. The fact that the ratio of the total fluorescence to tryptophan phosphorescence in HLAD is the same with excitation by 280-nm light as with 300-nm light indicates that the fluorescence heterogeneity arises solely in the tryptophan fluorescence spectrum, as opposed to different tyrosine fluorescence contributions to the total HLAD emission at 290- and 305-nm excitation.

In general then, the tryptophan fluorescence spectrum in HLAD represents a composite of two distinct spectra from individual tryptophan residues. In a rigid glass different triplet and singlet energies from HLAD tryptophans can be observed in the fluorescence, phosphorescence, and phosphorescence excitation spectra of the enzyme. The exciting wavelength dependence for the fluorescence spectrum of HLAD observed at low temperatures largely disappears when the protein is in solution at room temperature and only a small residual fluorescence shift remains (Figure 4b). However, note that in contrast to the low-temperature fluorescence result, 305-nm excitation at room temperature yields a fluorescence spectrum which is slightly *blue shifted* relative to that obtained at 290-nm excitation. The interpretation of these data in terms of the relaxation of the mobile

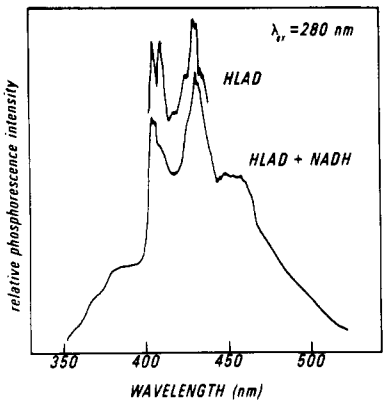

FIGURE 5: Phosphorescence spectra of 10^{-4} M HLAD in a 1:1 ethylene glycol–buffer glass at 77°K with 1:1 equiv of NADH and (insert) HLAD alone. Exciting wavelength is 280 nm. Emission below 400 nm is due to tyrosine.

environment about an exposed excited tryptophan will be presented in the Discussion.

Characterization of the Tryptophan Heterogeneity in HLAD. Heavy atoms or heavy atom containing compounds are known to perturb excited state processes in aromatic molecules with which these perturbers come into close proximity (McGlynn *et al.*, 1962). In tryptophan the presence of bromide ion or iodide ion causes quenching of the fluorescence (Konev, 1967; Lehrer, 1967) and an enhancement in the phosphorescence, primarily through an increase in the efficiency of singlet–triplet intersystem crossing (R. M. Purkey and W. C. Galley, unpublished results). Moreover bromide ion reduces the tryptophan phosphorescence lifetime from 6 sec to about 1 sec.

In a glass containing 3 M sodium bromide,[2] the tryptophan phosphorescence decay curves for either the 405- or 410-nm HLAD emission components, excited at wavelengths varying from 280 to 305 nm, were resolvable into a sum of two exponential decays, with lifetimes of *ca.* 5 and 1 sec, respectively. Relatively more short-lived phosphorescence decay was found for the 405-nm tryptophan 0–0 peak than for the 410-nm peak. This suggests that the 405-nm tryptophan is more exposed to the solvent, hence more readily accessable to the bromide ion than the 410-nm tryptophan. The similarity of the excitation spectrum for the 405-nm tryptophan to that of free tryptophan methyl ester and their identical short-wavelength phosphorescence maxima at 405 nm corroborate this conclusion.

The position of the 410-nm tryptophan peak in HLAD appears to derive from the greater polarizability of the environment of a tryptophan embedded in the protein core. In support of this hypothesis it is found that the phosphorescence maximum of tryptophan methyl ester is shifted from 405 nm in ethanol (n_D = 1.36) to 408.5 nm in much more polarizable 1,3-dibromo-2-propanol (n_D = 1.55). Similarly, the phosphorescence maximum of indole is shifted from *ca.* 402 nm in either ethanol or 1:1 ethylene glycol–water to 406 nm in a polystyrene film.

In summary, the heterogeneity in absorption and emission spectra for tryptophans in HLAD arises from variations in the local environment of individual tryptophan residues. Two types of tryptophan microenvironment are indicated by our phosphorescence data: a solvent-exposed, polar environment and a relatively less polar, but more polarizable environment in the interior of the protein. Denaturation of HLAD in 3 M guanidine hydrochloride and 10^{-1} M mercaptoethanol (Green and McKay, 1969) removes the environmental heterogeneity, resulting in a single, broadened phosphorescence peak at 410 nm.

Quenching of HLAD Emission by NADH. Upon binding of the coenzyme NADH to HLAD, the phosphorescence and fluorescence of the protein in a 1:1 ethylene glycol–buffer glass at 77°K are quenched to the same extent. This indicates that the quenching is occurring only at the singlet level and a concomitant enhancement in the ultraviolet-induced NADH fluorescence reveals that the quenching is a result of singlet–singlet nonradiative energy transfer from tryptophan to NADH. This is in agreement with the results of Velick (1961) who initially observed energy transfer to NADH in a number of dehydrogenases. Figure 5 displays the tryptophan phosphorescence spectrum of HLAD with and without NADH. It is apparent that the 410-nm peak is quenched to a greater extent by NADH than is the 405-nm peak.

At 290-nm excitation, the protein phosphorescence intensity at 405 nm is essentially due to the short-wavelength tryptophan in HLAD, plus a small contribution (<10%) from tyrosine and overlapping long-wavelength tryptophan. Therefore, measurement of the HLAD phosphorescence intensity at 405 nm under 290-nm excitation in the presence and absence of NADH gives directly the degree of quenching of the emission of the exposed tryptophan by NADH. After a small correction for the exciting light absorbed by NADH, 23 ± 5% NADH quenching of the emission from this tryptophan is found. Since upon 305-nm excitation only the long-wavelength tryptophan is observed in the HLAD phosphorescence spectrum, the degree of NADH quenching for this tryptophan can also be directly estimated and is found to be 71 ± 12%. Alternatively, decomposition of the HLAD phosphorescence spectrum at 290-nm excitation, based on the known spectral distribution for tyrosine and the assumption that both tryptophan spectra have the same shape, yields 25 ± 5% and 72 ± 14% NADH quenching for the 405- and 410-nm tryptophans, respectively. *Hence, singlet–singlet energy transfer from individual tryptophans to NADH can be resolved in HLAD and different singlet energy-transfer efficiencies for the two distinct tryptophan classes are obtained.*

Even though the fluorescence spectra at room temperature for the two tryptophan classes in HLAD are practically coincident, the heterogeneity in the tryptophan absorption spectrum is expected to be preserved (see Discussion). Because the tryptophan excited exclusively at 305 nm was found to possess a higher singlet-transfer efficiency to NADH in the phosphorescence spectrum of HLAD it is anticipated, and in fact observed, that a larger fraction of tryptophan room-temperature fluorescence from HLAD is quenched by NADH with excitation at 305 nm than at 290 nm. Quantita-

[2] Due to the absence of collisional perturbation through diffusion, a high density of bromide ion is required in the rigid glass matrix to ensure that tryptophan and perturber molecules achieve the proximity necessary for interaction.

tive measurements are difficult to make since at the concentrations necessary to ensure complete binding of all HLAD by NADH, large amounts of exciting light and HLAD fluorescence are trivially absorbed by NADH in the sample. Alternatively, we have measured the NADH quenching of tryptophan fluorescence and phosphorescence from HLAD in the ternary complex with excess isobutyramide. According to Yonetani and Theorell (1962) the dissociation constant for the ternary complex is *ca.* 100 times less than that for the binary NADH–HLAD complex. With 305-nm excitation 70% quenching of tryptophan fluorescence at room temperature by NADH was found in the ternary complex, in good agreement with the 73 ± 10% figure obtained from the phosphorescence data at low temperatures. Furthermore, the *total* quenching of HLAD phosphorescence at 290-nm excitation by NADH in the ternary complex was found to be 58 ± 8%, in excellent agreement with the result from the room-temperature fluorescence spectrum, namely, 57%. This last result indicates that the ratio of phosphorescence-to-fluorescence efficiencies is the same for either tryptophan class.

Selective Triplet–Triplet Energy Transfer in HLAD. When the triplet–triplet sensitizer (Galley and Stryer, 1968), α-bromoacetophenone is bound to HLAD, apparently to one or more sulfhydryl groups in the protein, and excited with 330-nm light, it transfers a portion of its triplet energy to a proximal tryptophan. Though the energy transfer is inefficient, slow rotation of the phosphoroscope chopper eliminates most of the residual short-lived acetophenone phosphorescence to permit resolution of the sensitized tryptophan spectrum. Figure 6 presents this tryptophan spectrum which is seen to be exclusively that of the long-wavelength tryptophan class in HLAD, indicating that triplet–triplet energy transfer occurs selectively to one of the two types of tryptophan residues in the protein.

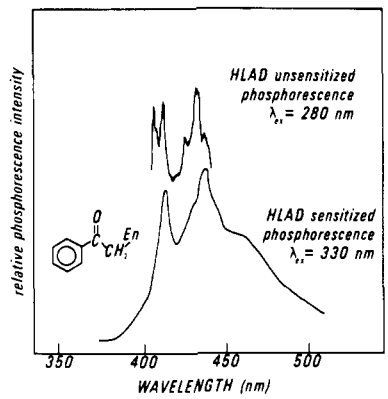

FIGURE 6: Tryptophan-sensitized phosphorescence spectrum from phenacylated HLAD (2×10^{-4} M) in a 1:1 ethylene glycol–buffer glass at 77°K, excited with light not directly absorbed by tryptophan. The insert displays the normal HLAD phosphorescence at 280-nm excitation for comparison. Sensitized HLAD phosphorescence is slightly distorted due to residual phenacyl phosphorescence.

Discussion

In the present study we have been able to establish from observations of the tryptophan phosphorescence of several proteins that heterogeneity exists in the electronic structure of the tryptophans within a given protein. This has been possible because of the vibronic structure which often appears in phosphorescence, but is absent in the corresponding fluorescence and absorption spectra. The appearance of individual vibronic bands in the tryptophan phosphorescence makes it possible to readily observe heterogeneity, such as the splitting seen with HLAD, in protein phosphorescence spectra.

The exciting wavelength dependence of the protein phosphorescence spectrum has been used to advantage to reveal that the heterogeneity in the tryptophan phosphorescence arises from emission from independent tryptophans, or classes of tryptophans, having distinct absorption spectra. Utilizing this difference in absorption for the two classes of tryptophans, we have been able to demonstrate a corresponding difference in the fluorescence spectrum for tryptophans in proteins in rigid media. An exciting wavelength dependence for protein fluorescence has been described previously (Weinryb and Steiner, 1970), but the observations were made at shorter exciting wavelengths and the difference in fluorescence attributed to contributions to interior tryptophan emission from tyrosine through singlet energy transfer.

The variations in the tryptophyl electronic energies which result in the spectral heterogeneity arise from perturbations due to the particular local environment about each of the tryptophans in the native molecule. In HLAD there are two distinct tryptophans in the monomer unit. The phosphorescence data suggest the occurrence of these in different local environments is the source of the spectral heterogeneity for this enzyme. The major factor contributing to this heterogeneity appears to be the relative accessibility of the two tryptophans to the polar solvent. This conclusion, based on our phosphorescence data, is in agreement with the earlier conclusions arrived at by Konev (1967) from the room-temperature fluorescence spectra of a number of proteins. In a recent report Teale and Badley (1970) point out that their data on tryptophan fluorescence quenching with iodide ion in pepsinogen is consistent with a buried residue having a long-wavelength absorption. This suggestion is confirmed in the present study in which we have shown that the class of tryptophans absorbing at longer wavelengths corresponds to residues within the interior of proteins.

The Model. The observations of spectral heterogeneity we have made, along with those of earlier workers, can be understood qualitatively in terms of a model which emphasizes the interaction of a polar molecule with its environment. The positions of the phosphorescence peaks at 405 and 410 nm are the result of the relative effect of environmental polarity, mobility, and polarizability on the excited and ground state energies of an "isolated" tryptophyl residue, that is, a hypothetical molecule *in vacuo*. If this hypothetical isolated tryptophan is placed in a polar solvent, favorable orientation of the solvent dipoles with respect to the dipole in the tryptophan lowers its ground-state energy. The same solvent orientation will not be as favorable for this tryptophan in its excited state where its dipole moment is different in magnitude and/or direction. Recognizing that the Franck–Condon principle must apply to a molecule plus its environment, that is, all the nuclei remain fixed on excitation, the

absorption spectrum for tryptophan in a polar solvent will be blue shifted relative to the isolated molecule (Bayliss and McRae, 1954). In a *rigid medium*, the orientation of the environment is maintained so that there is also a corresponding blue shift in the fluorescence and phosphorescence spectra.

If the environment of a tryptophan is nonpolar, albeit polarizable, the interactions will be dipole-induced dipole in nature. For an isotropically polarizable medium these interactions are completely independent of orientation. Both the excited and ground-state energies are lowered by the interaction. With an increase in the magnitude of the dipole moment in the excited state, the energy of that state will be lowered more than the ground-state energy. This produces a red shift in the absorption and emission spectrum, again relative to the isolated molecule. It is this type of interaction we maintain which results in the 410-nm component of the tryptophan phosphorescence in proteins.

When the *polar* solvent is not rigid, reorientation of the solvent dipoles about a chromophore following its excitation will lead to a lowering of the excited state and an increase in the energy of its ground state. If the reorientation can occur in a time which is short compared to the excited state lifetime, the well-known red shift in the emission observed for many molecules in polar solvents will occur. This effect has been treated by Lippert (1957) and we merely wish to emphasize the importance of solvent mobility in the production of the red shift. An environment which is polar, albeit rigid, during the lifetime of the excited state will not show the red shift characteristic of polar, mobile solvents. These considerations apply to the fluorescence of the tryptophyl residues exposed to the polar solvent, which at low temperature absorb and emit to the blue of the buried residues. At room temperature the tryptophan excited state is lowered by solvent relaxation, and the tryptophan fluorescence from the exposed residue in HLAD which is excited at shorter wavelengths is now slightly red shifted, relative to the fluorescence excited at 305 nm. This is consistent with previous observations (Lehrer and Fasman, 1967; Lehrer, 1967; Elkana, 1968) which have indicated a blue shift in protein fluorescence spectra with quenching of exposed tryptophans. It is interesting to note that *at room temperature* the solvent exposed tryptophans emit to the red but absorb to the blue of the buried residues.

Energy Transfer. The ability to selectively excite and recognize in emission, individual tryptophans or classes of tryptophans in a protein, enhances the usefulness of energy transfer involving tryptophan as a probe of protein structure and function. The observation in itself of more than one emitting tryptophan species in the protein phosphorescence requires that transfer between the two types of tryptophans cannot be highly efficient at either the triplet or singlet level. Inefficient transfer between tryptophans in proteins has been reported a number of times (Lehrer and Fasman, 1967; Galley and Stryer, 1969), and is anticipated on theoretical grounds (Eisinger *et al.*, 1969).

Förster's treatment of nonradiative energy transfer relates the transfer efficiency to the distance between donor and acceptor and their relative orientation. That the two tryptophans in HLAD exhibit different transfer efficiences to the singlet acceptor NADH, implies that the two tryptophans differ in distance from, and/or orientation of their excited singlet transition moments with respect to, NADH. A calculation (Förster, 1966) is made of $r/\kappa^{1/3}$, where r is the tryptophan–NADH distance and κ is a factor expressing the relative orientation of the donor and acceptor transition dipole moments. Using corrected tryptophan fluorescence and NADH absorption spectra and assuming a medium index of refraction of 1.5 a tryptophan fluorescence efficiency of 0.15 and singlet-transfer efficiencies of 0.25 and 0.70 for the 405- and 410-nm tryptophans, respectively, we obtain 21 Å for the $r/\kappa^{1/3}$ value of the 410-nm tryptophan and 27 Å for the 405-nm tryptophan in the binary HLAD–NADH complex.[3] The observation that the transfer efficiencies for the two tryptophans are essentially the same in the glass at 77 °K and at room temperature suggests that the conformation of the enzyme is similar under the two conditions.

Triplet–triplet energy transfer occurs *via* an exchange interaction requiring wave function overlap between donor and acceptor (Ermolaev, 1963). Because of this proximity requirement it is anticipated (Galley and Stryer, 1968) that transfer from an extrinsic probe would in most cases involve not more than one of the tryptophans in a protein. This is borne out in the case of HLAD in which triplet energy transfer from the phenacyl group can be recognized as occurring to the buried residue.

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N-Formylmethionyl Transfer Ribonucleic Acid in Mitochondria from *Neurospora**

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ABSTRACT: Although the mechanism for the initiation of protein synthesis in bacteria appears to involve fMet-tRNA, little is known of the initiation process in higher organisms. Since the mitochondria of *Neurospora* contain a protein-synthesizing system with components distinct from those of the cytoplasm, we have examined both mitochondrial and cytoplasmic extracts for fMet-tRNA. A Met-tRNA from the mitochondria can be formylated with an *Escherichia coli* extract, but the corresponding cytoplasmic tRNA cannot be. This mitochondrial fMet-tRNA is chromatographically separable from another mitochondrial Met-tRNA, which cannot be formylated. These are different from the cytoplasmic Met-tRNA and *E. coli* Met-tRNAs. The mitochondrial Met-tRNA synthetase is specific, aminoacylating only the mitochondrial tRNAs and not the cytoplasmic. Similarly, the mitochondrial extracts contain a formylase which reacts only with the mitochondrial fMet-tRNA. Thus, it appears that mitochondrial protein synthesis involves a formylated methionine, presumably as an initiator in analogy to the bacterial system.

The isolation of fMet-tRNA (Marcker and Sanger, 1964; Marcker, 1965) in bacteria and the elucidation of its role as an initiator of protein synthesis (Clark and Marcker, 1966; Adams and Capecchi, 1966) have resulted in a search for a similar system in numerous other organisms. In their early work Marcker and Sanger (1964) reported the presence of *N*-formylmethionine in yeast but not in various mammalian tissues. Similarly, Clark and Marcker (1966) examined mammalian tissues with no success, although Noll (1966) was able to detect the compound in rabbit reticulocytes. Caskey *et al.* (1967) showed that one of two Met-tRNAs from guinea pig liver could be formylated with a preparation from *Escherichia coli*. However, the enzymatic formylation could not be carried out by the liver extract. Recently, Li and Yu (1969) reported both fMet-tRNA and a formylase from Ehrlich ascites cells.

Since mitochondria appear to contain a protein-synthesizing system (*e.g.*, Wintersberger, 1965) distinct from that of the cytoplasm and more bacteria like in nature, a number of investigators have examined organelles for the presence of formylmethionine. Smith and Marcker (1968) showed that fMet-tRNA can be detected in yeast and in rat liver mitochondria, while Galper and Darnell (1969) showed that exclusively the mitochondria of Hela cells contain fMet-tRNA. In addition, the observations of Schwartz *et al.* (1967) suggest that initiation in *Euglena* chloroplasts utilizes *N*-formylmethionine.

Since the mitochondrion of *Neurospora* employs a protein-synthesizing system with components distinct from those of the cytoplasm (Barnett and Brown, 1967; Barnett *et al.*, 1967; Dure *et al.*, 1967), we have examined both mitochondrial and cytoplasmic extracts for fMet-tRNA. We show here that mitochondrial extracts contain a fMet-tRNA and the enzyme that carries out the formylation, whereas cytoplasmic extracts do not.

Materials and Methods

Strains. Neurospora crassa wild-type strain OR23-1A was used. *E. coli* strain A-19 was used in the preparation of bacterial extracts.

Preparation of Mitochondrial and Cytoplasmic Fractions. Hyphas from cultures grown 24–36 hr in aerated flasks of enriched Vogel's (1956) medium were harvested on cheesecloth and washed with cold distilled water. Fractions were prepared as described by Epler (1969) with slight modifications. In a standard preparation of 1500 g (wet weight), each of six 250-g mycelial pads was homogenized with 900 ml of grinding medium (0.005 M EDTA, adjusted to pH 7.5, 8%

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