SINGULAR OXYGEN EFFECTS ON THE ROOM-TEMPERATURE PHOSPHORESCENCE OF ALCOHOL DEHYDROGENASE FROM HORSE LIVER

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ABSTRACT The room-temperature phosphorescence of alcohol dehydrogenase from horse liver in the presence of oxygen has the characteristics of a light-induced emission as it appears only after a certain amount of excitation has been absorbed. The initial lag period, the steady-state intensity, and the duration of the induced state are dominated by the oxygen content of the solution, the rate of light absorption, and the radiation dose. The phenomenon is not unique to this enzyme and the data are consistent with photochemical depletion of oxygen.

INTRODUCTION

Information on the flexibility of protein structure has often been deduced from the ability of oxygen to quench the emission of tryptophan (Trp) residues buried within the folds of the polypeptides. Lakowicz and Weber (1973), from the fluorescence quenching of several globular proteins, concluded that rapid fluctuations in the protein structure generally occur on the nanosecond time scale allowing oxygen diffusion to the innermost chromophores. On the other hand, quenching of the steady-state phosphorescence from the buried Trp 314 in liver alcohol dehydrogenase from horse (LADH) is not consistent with such a model as this, and with other enzymes the access of oxygen to buried chromophores was deemed to be severely restricted (Saviotti and Galley, 1974). More recently, Vanderkooi et al. (1982) assert that oxygen can reach Trp 314 at a rate within an order of magnitude of the diffusion controlled rate. In the present investigation the room-temperature phosphorescence of LADH with continuous excitation was analyzed in detail to better elucidate the quenching role of oxygen. Rather surprisingly this emission was found to appear only after a relatively large amount of excitation had been absorbed by the sample. Such light-induced phosphorescence is interpreted as arising from photochemical depletion of oxygen from the solution.

MATERIALS AND METHODS

Alcohol dehydrogenase from horse liver (crystallized and lyophilized) and alkaline phosphatase, type III, from *Escherichia coli* were purchased from Sigma Chemical Co. (St. Louis, MO). These preparations were used without further purification. All samples were prepared in pyrophosphate buffer 30 mM, pH 8.6. The oxygen content of protein solutions was controlled by bubbling the solvent with a mixture of oxygen and nitrogen of known composition (SIO, Florence, Italy). After allowing 20 min for equilibration, we dissolved a weighed amount of dry protein powder in the same container. Oxygen partial pressures were converted to concentrations using Henry's Law and the solubility of oxygen in water at 25°C.

Luminescence measurements were carried out with a conventionally designed phosphorimeter fluorimeter. Exciting light was provided by a 100 W high-pressure mercury arc (HBO 100 W/2; Osram Sales Corp., Newburgh, NY) and was filtered through a Ni-Co sulphate solution to which benzoic acid was added such that ultraviolet radiation below 290 nm was effectively cut off. The excitation was further selected by an 100-mm grating monochromator (H10; Jobin-Ivon, Longjumeau, France) centered at 300 nm with a band pass of 10 nm. For experiments requiring intense excitation, the monochromator was removed. Variation in the exciting light intensity was achieved by ultraviolet neutral density filters (Oriel Corp. of America, Stamford, CT). As excitation, fluorescence, and phosphorescence spectra remained unaltered for all experimental conditions employed, intensity measurements were carried out at the same wavelengths, 350 nm for fluorescence and 400 nm for phosphorescence.

Phosphorescence decays were monitored by a double-shutter arrangement. The decaying emission was stored and averaged in a Varian C-1024 time-averaging computer (Varian Associates, Inc., Palo Alto, CA). 10 to 30 decays were sufficient to give a good signal-to-noise ratio. The steady-state phosphorescence intensity varies with the amount of exciting light absorbed by the sample, intersystem crossing efficiency, and geometrical factors due to the optical density of the solution. To analyze phosphorescence intensities of samples with different protein concentrations and oxygen content in terms of triplet-state lifetimes only (Domanus et al., 1980), a normalization must be carried out to account for the above mentioned contributions. This correction was achieved upon dividing phosphorescence intensities by the corresponding fluorescence signal.

The duration of the light-induced phosphorescent state, expressed as the memory time (MT), was obtained by exciting the sample for ~0.7 s at intervals from a few seconds to minutes as deemed appropriate from previous trial runs. Overestimations occur when following this procedure, but should not exceed 20%. The data referred to as radiation dose were obtained in the following way; the sample was excited for 3 min during which time we recorded the phosphorescence intensity and lifetime. Afterwards, the solution was gently stirred and left for 2 min to equilibrate before starting a new run. Protein samples for luminescence measurements were contained in cells made of spectrosil quartz tubing, 4-mm id. The temperature was kept constant at 25 ± 0.5°C.

RESULTS

The room-temperature phosphorescence of LADH in air equilibrated solutions, as detected by the conventional

phosphorimeter, is singular in that it appears only after several seconds of excitation. Following a lag period of undetectable levels of phosphorescence, the triplet-state emission grows rapidly in a distorted sigmoidal manner to remain constant at its steady-state value except for a gradual decrease in intensity as photochemistry proceeds. When the exciting light is interrupted, the emission decays rapidly according to the triplet-state lifetime, but the light-induced molecular state responsible for the phosphorescence lasts from seconds to minutes. The process and the parameters are shown schematically in Fig. 1.

Varying Oxygen Concentration

Fig. 2 shows how all parameters are strongly affected by the presence of oxygen. The induction time (*IT*) decreases monotonically with lower oxygen content. It does not, however, reach zero even with the most rigorous deoxygenating procedures. This might reflect the difficulty of totally abstracting O_2, particularly with protein solutions where the hydrophobic cores of the macromolecules may provide sites of high solubility if not weak associations (Lower and El-Sayed, 1966). Oxygen-saturated solutions, on the other hand, will not give detectable levels of phosphorescence even after prolonged excitation.

MT becomes shorter in the presence of O_2. In deoxygenated solutions they are long, and barely measurable, dropping to a few seconds in the opposite end of the concentration range. The triplet-state decay is monoexponential only in oxygen-free solution with a lifetime, τ_p, of 120 ± 5 ms. In the presence of O_2 the decay becomes more rapid and highly nonexponential. By fitting it as a sum of two exponentials, for the purpose of obtaining lifetime independent phosphorescence intensities, I_p/τ_p, more than 90% of the emission can be accounted for.

A large decrease in the ratio of the steady-state phosphorescence intensity, I_p, to the triplet lifetime, τ_p, with increasing O_2 concentration is also seen in the Fig. 2. If O_2 influenced the phosphorescence by simply shortening τ_p through a dynamic triplet quenching, the ratio I_p/τ_p would be invariant to O_2 concentration. This follows from the relations

$$I_p = \Delta I \cdot \phi_p,\ \phi_p = k_p \cdot \tau_p \cdot \phi_{isc},\ \Delta I = I_f/\phi_f,$$

where I is the rate of light absorption, ϕ's are quantum efficiencies, k_p is the triplet-radiative rate constant, and the subscripts p and f indicate phosphorescence and fluorescence, respectively. With appropriate substitution one obtains

$$\frac{I_p}{\tau_p} = k_p \cdot \frac{\phi_{isc}}{\phi_f} \cdot I_f.$$

The fluorescence-normalized ratio, I_p/τ_p, should be invariant to O_2 assuming that k_p is unperturbed and that at the O_2 concentrations employed, the singlet parameters, ϕ_{isc} and ϕ_f, are also not influenced by O_2. Hence the remarkable drop in I_p/τ_p with increasing O_2 reveals that within the sample volume being excited, only a fraction of the macromolecules are able to contribute to the overall phosphorescence.

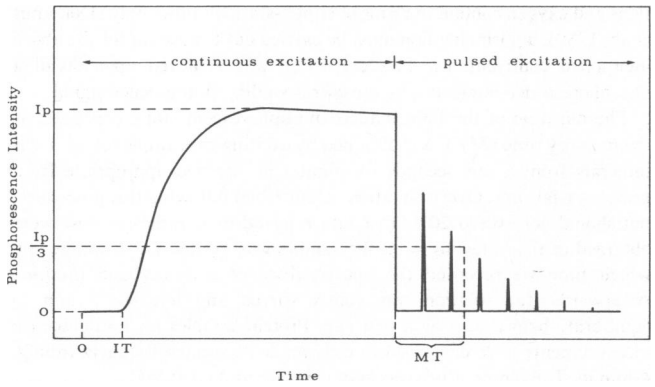

FIGURE 1 Schematic representation of the room-temperature phosphorescence intensity of LADH in buffer as a function of time. The parameters I_p, *IT*, and *MT* refer to the steady-state phosphorescence intensity, induction time, and memory time, respectively.

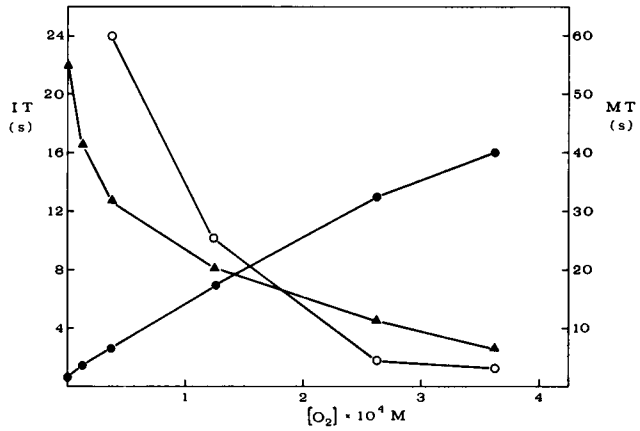

FIGURE 2 Dependence of the parameters *IT* (●), I_p/τ_p (▲), and *MT* (○) upon the oxygen concentration of the solution. LADH concentration is 100 μM.

Effects of Protein Concentration

Concentration effects on luminescence are often an indication that either chromophores are fairly close to interaction with one another or that they are part of a chemical equilibrium. Fig. 3 reports a remarkable concentration dependence on the parameters describing the phosphorescence of LADH in air equilibrated solutions. Increasing protein concentration leads to smaller values of *IT*, larger intensities, and longer *MT*, effects previously noted in reducing the amount of oxygen in solution. Furthermore, one finds that the product (*IT*) · [protein] is roughly constant. A similar relationship is also obeyed between *IT* and the intensity of the exciting light, I_0. In general then

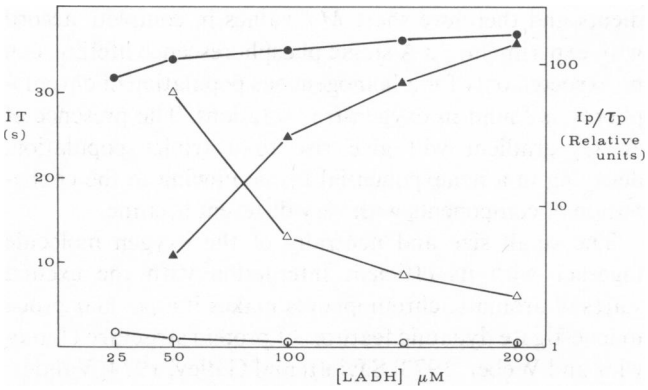

FIGURE 3 Influence of protein concentration upon the parameters describing the induced room-temperature phosphorescence of LADH: *IT* (△) and I_p/τ_p (▲) in air equilibrated samples; *IT* (○) and I_p/τ_p (●) in deoxygenated samples.

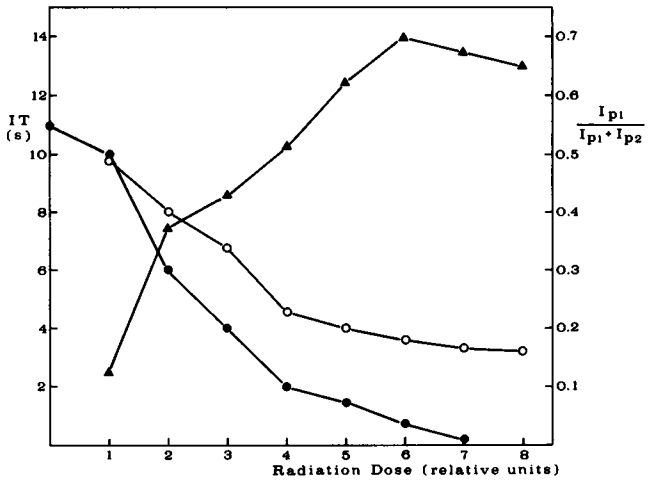

FIGURE 4 Effect of radiation dose upon *IT* (●), I_p (▲), and the fraction of the steady-state phosphorescence intensity decaying with a lifetime of 18 ± 5 ms (○). LADH concentration is 100 μM.

one can write

$$IT \alpha\ 1/[\text{protein}] \cdot I_0.$$

For small optical densities, the product (I_0) · (absorbance) represent the rate of light absorption. The above relationship then implies that induction always take place after the same amount of light has been absorbed by the sample. This amount is governed entirely by the oxygen content of the solution. Deoxygenated samples have a typical lifetime of 120 ± 5 ms, a small nonzero *IT* and I_p/τ_p that does not remain constant with protein concentration although its variation is minimal compared with that observed in the presence of O_2. Both oxygen traces and phosphorescence enhancement by trivial singlet-singlet energy transfer between tryptophans, not corrected for by fluorescence normalization, might be responsible for this increment in I_p/τ_p.

Effects of Radiation Dose

If oxygen mediated photochemistry is responsible for the induced phosphorescence, the effect of the amount of light absorbed by the system and the reversibility of the process require investigation. Fig. 4 reports such a study in samples open to the atmosphere. All parameters are affected by dose. Concomitant to a decrease in *IT* upon repeated irradiation, there is a fivefold increase in phosphorescence intensity. Eventually a plateau is reached after which no further variation occurs. When the decay in the triplet-state population is analyzed in terms of a short- and a long-lived component, one finds lifetimes of 18 ± 5 ms and 85 ± 5 ms, respectively. The contribution of the short-lived component to the total emission goes from ~50% for the first run to 16% at the plateau. As before, the larger triplet-state population with dose is not compensated by a slower decay. Dose effects are completely reversible if the sample is allowed to re-equilibrate with air. Such reversibility is a further indication that permanent photochemistry on the macromolecules, if it does take place, does not induce the phosphorescent state. No dose effect is found for deoxygenated solutions.

DISCUSSION

In a steady luminescence experiment the emission intensity builds up from the beginning of excitation to reach a plateau in a $1 - \exp(-t/\tau)$ manner, τ being the emitting-state lifetime. The room-temperature phosphorescence of Trp 314, placed in the deep interior of the globular subunits of LADH, responds to excitation in an unusual fashion. As this behavior is found for a wide range of protein concentrations, down to the micromolar, as well as low levels of excitation intensities smaller than normally employed in phosphorescence studies, the possibility of an artifact arising from local heating of the sample and/or protein denaturation must be ruled out.

The presence of oxygen in solution plays a key role in this phenomenon as abnormalities are almost entirely restored upon degassing. The interaction of oxygen with excited triplet states of aromatic molecules leads to the formation of excited singlet oxygen, a very reactive species yielding photoperoxidation (Kearns, 1971). Particularly efficient oxygen depletion is therefore anticipated with long-lived Trp triplet states to which oxygen can diffuse within interaction distance. Furthermore, the process is cooperative in that lengthening the triplet lifetime lowers O_2 concentrations which in turn lengthens the triplet lifetime. Tryptophans buried in the deep hydrophobic interior of some proteins have phosphorescence lifetimes in fluid solutions up to four orders of magnitude larger (Saviotti and Galley, 1974) compared with the completely solvated chromophore (Bent and Hayon, 1975). Further, the variety of amino-acid side chains offer ample opportunity for oxidation reactions. On these grounds such pro-

teins are good candidates for effective O_2 depletion down to very small levels.

The observed effects of oxygen on the room-temperature phosphorescence are not confined to LADH. They have been observed and are presently studied with alkaline phosphatase and would seem of a rather general nature. The simplest picture consistent with experimental data shows the diffusion of oxygen within the macromolecule, its depletion through sensitization via the long-lived triplet state of the internal Trp, which results in the establishment of a concentration gradient perpendicular to the direction of the exciting light. No phosphorescence is detected at the onset of excitation and none is detected until the bottom of this gradient reaches oxygen concentrations not totally quenched. Upon reaching the steady state, only a fraction of excited macromolecules are found in a region of the beam with O_2 below such a critical concentration. This fraction contributes to the excited triplet-state population as detected by I_p/τ_p. On interrupting the excitation, oxygen diffusion inwards abolishes the concentration gradient completely restoring the initial O_2 quenching levels. *MT* values measure the rate of this process.

While a quantitative analysis has not been attempted, a satisfactory qualitative explanation can be advanced for all quantities observed. *IT:* As phosphorescence will be detectable only after the O_2 gradient reaches below the critical concentration, the *IT* will be longer the greater the initial O_2 content of the solution (Fig. 2) and the smaller the rates of excitation employed (Fig. 3). The effects of radiation dose come from a progressive reduction of oxygen content with successive runs to reach a minimum value determined by exchange with the atmosphere. Initial conditions are restored when the oxygen content is repristinated by air re-equilibration. I_p/τ_p: Lowering the O_2 content as well as increasing the rates of light absorption or radiation dose brings an ever greater proportion of macromolecules into an environment free from oxygen quenching, which will then contribute to the enhancement of the overall phosphorescence intensity. *MT: MT*, on the other hand, are characterized solely by the steepness of the oxygen gradient. This, in turn, is determined by the initial oxygen concentration as well as by the size of the light beam. High concentrations and small beam size make for steep gradients and therefore short *MT* values in complete accord with experiment. τ_p: A single phosphorescence lifetime can be expected only for a homogeneous population of chromophores as found in oxygen-free solutions. The presence of an O_2 gradient will give rise to a triplet population, decaying in a nonexponential fashion owing to the contribution of components with very different lifetime.

The small size and neutrally of the oxygen molecule together with its efficient interaction with the excited states of aromatic chromophores makes it a popular probe to investigate dynamic features of protein structure (Lakowicz and Weber, 1973; Saviotti and Galley, 1974; Vanderkooi et al., 1982). The present study indicates that oxygen quenching of luminescence may be subject to erroneous interpretations. Possible reasons are (*a*) poor control of the oxygen content due to its depletion as well as its unknown solubility within the macromolecule. The implications from the work of Saviotti and Galley (1974) on LADH must be reconsidered in this light. (*b*) The formation of singlet oxygen may result in peroxydation of the biopolymer with alteration of some of its structural features.

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