Photoreduction of Membrane Bound Cytochrome c by Excited-State Phenothiazine

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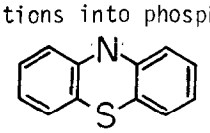
SUMMARY: Ferricytochrome c can be reduced in a photochemical reaction by excited state phenothiazine. This reaction is observed between phenothiazine which is solubilized by phospholipid artificial membranes and cytochrome c which is adsorbed to the membrane surface. Under conditions when cytochrome c is not bound to the phospholipid, the rate of reduction by phenothiazine is greatly reduced. The phosphorescence of phenothiazine is quenched in the presence of cytochrome c, implying that the excited triplet state interacts with cytochrome c. Oxygen inhibits the reaction since possibly, as a paramagnetic species, it increases intersystem crossing of the excited states of phenothiazine. On the basis of molecular models the proximity between the iron of ferricytochrome c and phenothiazine is estimated to be over 20 A.

Biological redox reactions are characterized by their speed and specificity. Although they provide most of the energy for the cell, and are therefore of utmost importance, a description of these reactions in molecular terms has been most difficult to formulate, arising in part because so many redox reactions occur within the membrane. Furthermore simple model systems for biological redox reactions are often not available. In the report given here a model system for the study of electron transfer reactions in cytochrome c is described. The reductant molecule is excited state phenothiazine (I). Phenothiazine is a small hydrophobic molecule which can be solubilized by detergents (1) and like similar molecules (2) partitions into phospholipid artificial membranes.



I

In its excited state it has been shown to reduce metals (1) and quinones

(3). Here data is presented showing that excited state phenothiazine can reduce ferricytochrome c in a phospholipid dispersion. The reaction is discussed in terms of a tunneling mechanism for electron transfer.

Materials and Methods:

Cytochrome c type VI, L-α-dimyristoyl lecithin and cardiolipin were obtained from Sigma Chemical Company (St. Louis, MO). Phenothiazine was supplied by Aldrich Chemical Company (Milwaukee, WI). Aqueous suspensions of phospholipids were prepared by sonicating the lipids for three minutes using a Branson sonifier at maximum output. Phenothiazine was sonicated with the lipids or small aliquots of phenothiazine added to the sonicated lipid suspension. A glass **cuvette** which was equipped with a screw cap and rubber septum through which a syringe could be inserted was used during the irradiation. Oxygen was first removed from the sample by bubbling the sample vigorously for five minutes with ultrapure nitrogen (Matheson Gas Company) and the sample was irradiated with a 100 Watt low pressure mercury arc lamp held at 12 inches from the sample.

Luminescence spectra were taken at liquid nitrogen temperature (77°K) using an Hitachi MPF-2A fluorescence spectrometer. The Hitachi phosphoroscope attachment and a quartz Dewar was used for the cell compartment. The phosphoroscope was used without the rotating shutter. Ferrocytochrome c concentration was determined using 27.6×10^{-3} M^{-1} cm^{-1} as extinction coefficient at 550 nm.

Results:

Photoreduction of cytochrome c: Irradiation of an anaerobic sample containing ferricytochrome c and vesicles composed of a mixture of cardiolipin, dimyristoyl lecithin and phenothiazine with light from a Hg arc lamp causes reduction of cytochrome c (Fig. 1). Prolonging the time of exposure to light produces more reduction and under the conditions described in the legend twenty minutes exposure resulted in about 30% reduction of cyto-

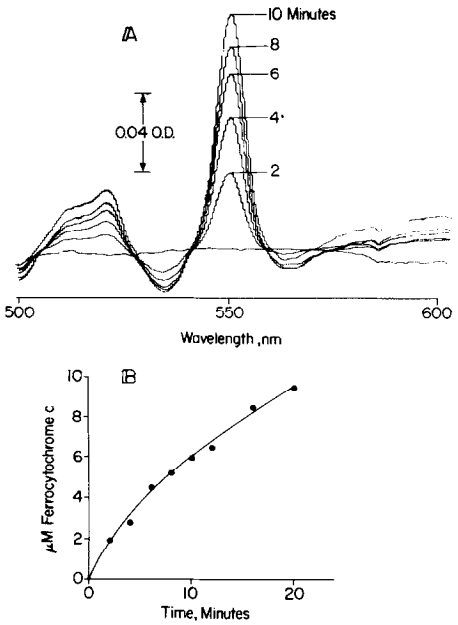


Figure 1: Photoreduction of cytochrome c: difference spectra before and after illumination. The sample contained mixed lipid vesicles composed of 0.5 mg dimyristoyl lecithin/ml., 0.2 mg cardiolipin/ml and 0.47 mM phenothiazine. Cytochrome c concentration was 24 μM and phosphate buffer (10 mM) was at pH 6.5. Sample prior to illumination was used as reference.

chrome c samples. The reaction rate decreases somewhat with longer times of exposure (Fig. 1b); a decrease in the rate is linked to the photodestruction of phenothiazine and to the lowering of the concentration of ferricytochrome c.

Several pieces of evidence indicate that excited state phenothiazine is the reductant. The reaction does not occur in the dark. Also, illumination of the reaction mixture in which phenothiazine is omitted produced no reduction of cytochrome c.

Under conditions identical to the experiment described in Figure 1 but in which atmospheric oxygen was not removed, cytochrome c was less than 1% reduced after 20 minutes exposure to light. The inhibition of the photoreduction of cytochrome c by oxygen may be related to several factors. First, irradiation of cytochrome c by ultraviolet light can produce a

photooxidation of cytochrome c. However, probably more importantly, molecular oxygen by virtue of its paramagnetism is expected to interact with excited states of phenothiazine to produce intersystem crossing as suggested by Alkaitis et al (1). These authors rule out the possibility that oxygen is itself reduced by phenothiazine in its excited triplet state.

The reduction rate of cytochrome c by excited state phenothiazine is greatly reduced when cytochrome c is not bound to the membrane. When 1.0 M KCl is substituted for 10 mM phosphate buffer, the reduction was inhibited (not shown). Under these conditions the negative charges on the surface of the artificial membrane are shielded and cytochrome c does not bind to the lipid (4). These data indicate that the reaction is occurring at the membrane interface and also rules out direct cytochrome c-phenothiazine interactions.

Luminescence spectra of phenothiazine. The inhibition of the reaction rates by oxygen would suggest that the long-lived triplet state of phenothiazine (7300 msec) (1) rather than the short-lived singlet state (< 2 nsec, our unpublished results) is the reactive species. If so, one might expect that cytochrome c would quench the phosphorescence of phenothiazine. This was indeed observed. In Fig. 2, emission spectra of phenothiazine in ethanol, mixed phospholipids and phospholipids to which cytochrome c is bound are presented. Light emitted from 400 to 480 nm is comprised of fluorescence and light emitted above 480 nm represents the phosphorescence.

Although the data presented by the Figure do not allow quantitative determination of the extent of quenching by cytochrome c, they do provide qualitative evidence that cytochrome c quenches the triplet of phenothiazine. Taking 462 nm as the wavelength for fluorescence which is least affected by scattering and absorption artifacts and 502 nm as the wavelength for phosphorescence, the intensity of phosphorescence to fluorescence is 17.6

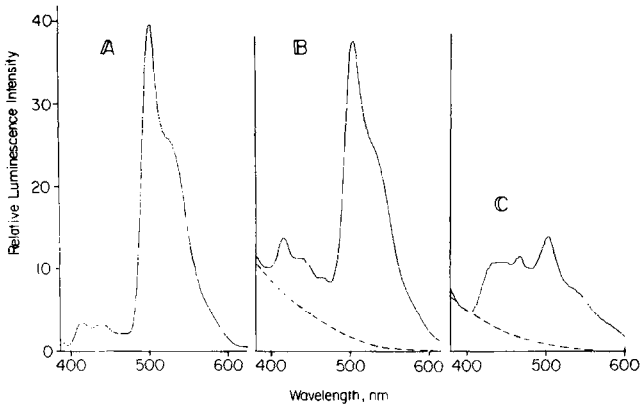


Figure 2: Luminescence spectra of phenothiazine at 77°K. A. 10 mM phenothiazine in ethanol B. 0.5 mg dimyristoyl lecithin/ml 0.2 mg cardiolipin/ml and 0.10 mM phenothiazine in 6 mM phosphate buffer, pH 6.5 and 30% glycerol. C. Same as B but 0.1 mM ferricytochrome c was added. Instrumental gain for C was 3 times that of B. Dotted line represents approximate baseline.

and 8.6 for phenothiazine in ethanol and lipids, respectively. This compares with a ratio of 1.25 for the lipid system in which cytochrome c is bound (Figure 2C). The differential quenching of phosphorescence over fluorescence of phenothiazine is related to the longer lifetimes of the triplet state; experiments are now in process to determine the rate constants. It can be mentioned, however, that at room temperature the binding of cytochrome c to negatively charged phospholipid vesicles which are doped with phenothiazine results in very little fluorescence quenching. This is in contrast to the quenching of anthracene under similar conditions (4). Although anthracene and phenothiazine have similar emission spectra, anthrancene has a much longer fluorescence lifetime (∿ 10 nsec), and consequently energy transfer from the singlet excited state is more probable.

Discussion:

Cytochrome c, a positively charged molecule, adheres to the surface of the negatively charged phospholipid dispersions (4). Cytochrome c is about 30 Å in diameter and the heme is slightly off center (5), making the distance from the iron to the nearest edge of the protein to be about

12 Å. Phenothiazine is extremely water insoluble and is expected to partition into the hydrophobic region of the membrane. This means that the distance between iron and phenthiazine is perhaps an additional 8 Å distance apart; therefore, the distance between the reductant and the oxidant is roughly greater than 20 Å. The finding that the electron can be transferred over such large distances is consistant with proposed tunneling mechanisms for electron transfer (6,7). Using the analogy that electron transfer and energy transfer can be described by similar equations (7), one would expect that the electron transfer like energy transfer is orientation as well as distance dependent. If however the electron is being transferred from the triplet state to the porphyrin ring system, the orientation factor for electron transfer, like energy transfer (8), should never go to zero. This is due to the symmetry of the ring.

The reaction system described here is an excellent model for the photosynthetic systems of plants and bacteria. Since the reductant can be instantaneously produced by a flash of light, it provides a potentially useful way to measure cytochrome c reaction kinetics. We are currently measuring the reaction rates of the fluorescence and phosphorescence of phenothiazine as well as the reduction rates of cytochrome c in order to get a detailed understanding of this redox reaction.

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References:

1. Alkaitis, S.A., Beck, G. and Gratzel, M. (1975) J. Am. Chem. Soc. 97, 5723-5729.

2. Vanderkooi, J.M., Fischkoff, S., Andrich, M., Podo, F. and Owen, C.S. (1975) J. Chem. Phys. 63, 3661-3666.

3. Alkaitis, S.A., Gratzel, M. and Henglein, A. (1975) Berichte der Bunsen Gesellschaft 79, 541-546.

4. Vanderkooi, J., Erecinska, M. and Chance, B. (1973) Arch. Biochem. Biophys. 113, 29-35.

5. Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, O.B., Samson, L., Cooper, A. and Margoliash, E. (1971) J. Biol. Chem. 246, 1511-1535.

6. DeVault, D. and Chance, B. (1966) Biophys. J. 6, 825-644.

7. Hopfield, J.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3640-3644.

8. Vanderkooi, J.M. and Erecinska, M. (1975) Eur. J. Biochem. 60, 199-207.