

Interaction of electron acceptors with the excited triplet state of Zn cytochrome *c*

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The decay rate of the excited triplet state of Zn cytochrome *c* was enhanced by electron acceptors including methyl viologen and ferric complexes of cyanide, oxalate, EDTA and cytochrome *c* at room temperature. Ferrous compounds were several orders of magnitude less effective than the respective ferric form in quenching the phosphorescence. In the presence of ferricytochrome *c* and ferricyanide the semilogarithmic plots of the decay curve showed an anomalous decay profile in which the rate of interaction appeared to accelerate after excitation. One explanation is that the quenching process was accelerated by a conformational change of the polypeptide chain around the excited triplet state porphyrin. Another explanation is that quenching occurs via an intermediate.

Cytochrome c Excited triplet state Electron acceptor Phosphorescence lifetime

1. INTRODUCTION

Photochemical redox reactions of porphyrins have been widely investigated as tools to examine the involvement of the porphyrin ring in electron transfer reactions of heme proteins [1,2]. Derivatives of the cytochromes in which the porphyrin moiety is luminescent can be obtained by substituting a variety of metals for the iron of the heme. In these derivatives the luminescence of the porphyrin can be used as a reporter of its role in electron transfer reactions.

Cytochrome *c* is one of the best studied heme proteins with regard to structure and electron transfer function [3]. Zn cytochrome *c*, in which Zn replaces the iron of the heme, exhibits phosphorescence at room temperature [4]. The longer lifetime of the phosphorescence compared with fluorescence can increase the possibility to detect the interaction between cytochrome *c* and other molecules and the perturbation of the microen-

vironment around Zn porphyrin within the polypeptide.

Here, the interactions between the excited triplet state of Zn cytochrome *c* with iron containing electron acceptors are presented.

2. MATERIALS AND METHODS

2.1. Materials

Horse heart cytochrome *c* (type III and type VI), potassium ferricyanide, glucose oxidase (type VII, from *Aspergillus niger*), catalase (bovine liver), and glucose were obtained from Sigma (St. Louis, MO); Rhodamine 575 was purchased from Exciton (Dayton, OH). Other reagents were of analytical grade. Zn cytochrome *c* was prepared according to the procedures of [5].

2.2. Triplet-triplet absorption and phosphorescence emission of Zn cytochrome *c*

The phosphorescence emission and absorption of the excited triplet state Zn cytochrome *c* were measured as in [6]. The sample for the measurement of the excited triplet state of Zn cytochrome *c* was deoxygenated by bubbling with ultrapure

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argon (Airco, Philadelphia) for 20 min in a glove bag. The sample contained 20 mM phosphate buffer (pH 7.4) containing glucose oxidase (75 μ g/ml), catalase (12.5 μ g/ml) and 0.3% glucose as in [7].

3. RESULTS

A variety of iron-containing compounds quenched the triplet state of Zn cytochrome *c* at room temperature. An example of a quenching profile is shown in fig.1 for Fe-EDTA. The lifetime for Zn cytochrome *c* phosphorescence in the absence of added quencher molecules was 14 ms. The semi-logarithmic plots of the decay of the triplet state of Zn cytochrome *c* in the presence of Fe-EDTA were linear, consistent with a reaction dependent upon collisions. Similar plots were obtained in the presence of Fe-oxalate and methyl viologen.

In the presence of ferricytochrome *c* or ferri-cyanide the quenching profiles of triplet decay for Zn cytochrome *c* were somewhat different from that described above. The semi-logarithmic plots of the decay of the triplet state of Zn cytochrome *c* bent downward in the presence of cytochrome *c* (fig.2) or ferricyanide (fig.3); such curves were observed both in the triplet-triplet absorption and phosphorescence emission.

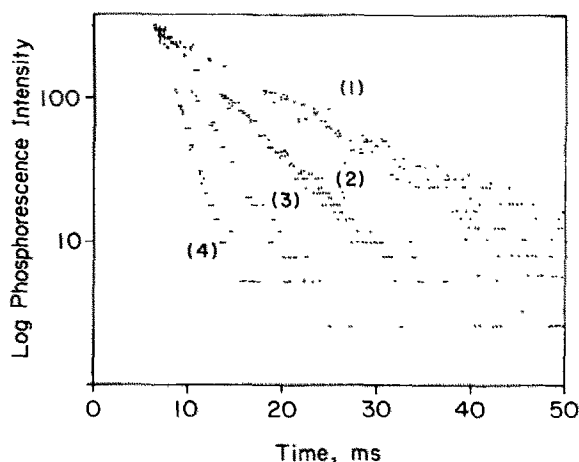


Fig.1. Semi-logarithmic plots of decay of excited triplet state Zn cytochrome *c*. Phosphorescence emission at 720 nm of Zn cytochrome *c* (15 μ M) in the presence of 10 mM EDTA, 10 mM phosphate buffer (pH 7.0) and FeCl₃ (1, none; 2, 100 nM; 3, 200 nM; 4, 400 nM) at 22°C.

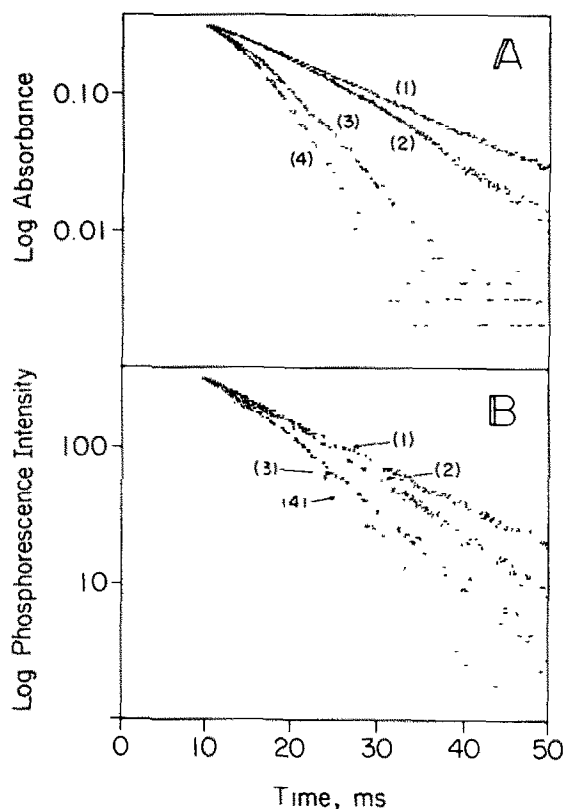


Fig.2. Semi-logarithmic plots of decay of excited triplet state Zn cytochrome *c* in presence of ferricytochrome *c*. (A) Triplet-triplet absorption at 460 nm, and (B) phosphorescence emission at 720 nm of Zn cytochrome *c* (20 μ M) in the absence and presence of native ferricytochrome *c* at room temperature. Cytochrome *c* (type IV): (1) none, (2) 10 μ M, (3) 20 μ M, (4) 40 μ M in A and 35 μ M in B, respectively.

The excited triplet state of Zn cytochrome *c* was quenched by both ferrocyanide and ferricytochrome *c* at concentrations about 100-times higher than for the ferri-compounds. The decay curves were also anomalous.

In table 1 the apparent bimolecular rate constants are given for the quenching of the triplet excited state of Zn cytochrome *c*.

To further characterize the quenching of Zn cytochrome *c* by ferricytochrome *c* we carried out the following experiments. The triplet-triplet absorption of Zn cytochrome *c* in the absence and presence of native ferricytochrome *c* was measured at different wavelengths, 430, 460 and 550 nm. The concentration dependency of Zn cytochrome *c* on

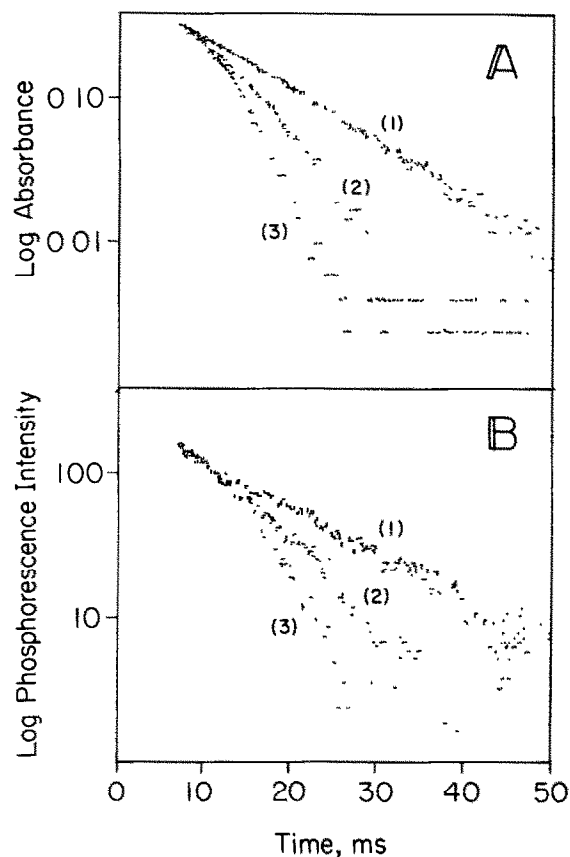


Fig.3. Semi-logarithmic plots of decay of excited triplet state Zn cytochrome *c*. (A) Triplet-triplet absorption at 465 nm, and (B) phosphorescence emission at 720 nm of Zn cytochrome *c* (20 μ M) in the absence and presence of ferricyanide at 5°C. Ferricyanide: (1) none (2) 20 nM (3) 60 nM.

the decay curves of the excited triplet state of Zn cytochrome *c* was investigated at 5, 10 and 20 μ M Zn cytochrome *c* with and without native ferricytochrome *c*. The data of the decay curves of the excited triplet state Zn cytochrome *c* were analyzed at different time scale with and without native ferricytochrome *c*. The concentration of the excited triplet state was varied by reducing the laser intensity with neutral density filters. To check for possible polarization effects, a polarizer was set for the excitation side of the samples and the excited triplet state of Zn cytochrome *c* was measured with and without native ferricytochrome *c*. The semi-logarithmic plots of decay curves of excited triplet state of Zn cytochrome *c* obtained from all of the

Table 1

Apparent quenching constants for excited triplet Zn cytochrome *c*

Quencher	l/mol per s
Ferric-oxalate ^a	3.7×10^7
Ferric-EDTA ^b	5.8×10^8
Potassium ferricyanide	1.3×10^8
Ferricytochrome <i>c</i>	2.4×10^6
Ferrocyclochrome <i>c</i>	1×10^4
Potassium ferrocyanide	2×10^6
Methyl viologen	1×10^7

The medium contained 10 mM phosphate buffer, pH 7
^aOxalate concentration, 10 mM; ^bEDTA concentration, 1 mM

experiments mentioned above gave similar anomalous decay curves. Furthermore, this anomalous decay curve was not influenced by increasing the ionic strength with addition of a small volume of concentrated KCl up to 0.6 M KCl final concentration. At the concentrations used there was no effect on the fluorescence intensity nor the steady-state absorption spectrum by any of the quenchers. These experiments allow us to conclude that there was no triplet-triplet interactions, no complex formation between quencher and ground state and no interaction between ground state Zn cytochrome *c* and the excited triplet state.

4. DISCUSSION

Excited triplet state Zn cytochrome *c* was quenched by iron-containing compounds and by methyl viologen.

Several mechanisms for the quenching of excited states are commonly evoked. These include: 1, dipole-dipole energy transfer; 2, electron exchange energy transfer; 3, electron transfer; and 4, enhanced radiationless decay. Dipolar energy transfer would appear to be unlikely because of the lack of overlap between the spectra of the Zn cytochrome *c* phosphorescence and ferricytochrome *c* absorption. However, the excited singlet state, which is thermally populated at room temperature [7], has spectral overlap with the ferricytochrome *c* absorption and therefore dipolar energy transfer from the triplet state could occur via the excited

singlet state. We feel that this mechanism of quenching is unlikely on the following grounds: 1, ground state Zn cytochrome *c* and ferrocytochrome *c*, both of which also have spectral overlap, quenched at much lower concentrations; and 2, overlap is not present for the other quenchers. Enhanced radiationless decay is unlikely because under the conditions of our experiments complexation does not occur. The most likely explanation of quenching is via the electron exchange and electron transfer mechanisms, both of which are spin allowed. No difference between absorption and emission profiles were observed; we therefore conclude that the electron transfer back reaction is very fast; no net electron transfer was detected by absorption measurement. We point out that authors in [8] have observed that cytochrome *b*₅ quenches the Zn cytochrome *c* triplet state and also conclude that electron transfer is a likely quenching mechanism and authors in [9] show electron transfer between Zn porphyrin and Fe porphyrin in hemoglobin.

The bimolecular rate constant for quenching was about the same for Fe-EDTA, Fe-oxalate and ferricyanide. These compounds are the same charge and have about the same size, but vary in redox potential from about +400 mV for ferricyanide to +0.02 mV for ferrioxalate. Methyl viologen, with midpoint redox potential of -400 mV, also quenched. If electron transfer is the mechanism for quenching, then we can conclude that the excited triplet state of Zn cytochrome *c* is a strong reductant.

The decay curves for Zn cytochrome *c* phosphorescence in the presence of ferricyanide and ferricytochrome *c* were anomalous: the quenching appeared to accelerate after the flash. This leads to speculation about the quenching mechanism. One possibility is that the polypeptide chain of cytochrome *c* rearranges around the excited Zn porphyrin and allows the quencher molecules access to the porphyrin. Another possibility is that electron transfer occurs via an intermediate in the polypeptide chain. For example, if the excited Zn porphyrin exchanges electrons with a neighboring amino acid and the quencher reacts with this moiety, then the curves would show increase rate with time. Or if either the quencher (or the protein) exists in two slowly interconverting forms and only one form quenches (or is quenched) such curves

may occur. Finally, another possibility is that each collision produces less than 100% quenching and that there are successive multiple collisions producing additional quenching.

We found that ferrous cytochrome *c* and ferrocyanide quenched the triplet state of Zn cytochrome *c* at concentrations which are about two orders of magnitude less than the respective ferri-compound. Excited states can act, in principle, either as oxidants or reductants: the excited electron is more easily lost than the ground state electron, but the hole left in the original orbital is more readily filled than the next higher vacant orbital. Therefore, we need to consider the possibility that the excited state can act as photooxidant as well as a photoreductant. However, because the reduced compounds quenched so much less effectively than the oxidized compounds, we cannot exclude the possibility that contamination of the ferro-compounds with a small amount of oxidized form could have produced the quenching of the excited triplet state of Zn cytochrome *c*.

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REFERENCES

- [1] Dolphin, D. and Felton, R.H. (1974) *Acc. Chem. Res.* 7, 26-32.
- [2] Mauzerall, D. (1975) in: *The Porphyrins* (Dolphin, D. ed.) vol. 5, pp. 29-51, Academic Press, New York.
- [3] Margoliash, E. and Bosshard, H.R. (1983) *Trends Biochem. Sci.*, 8, 316-320.
- [4] Dixit, B.P.S.N., Moy, V.T. and Vanderkooi, J.M. (1984) *Biochemistry* 23, 2103-2107.
- [5] Vanderkooi, J.M., Adar, F. and Erecinska, M. (1976) *Eur. J. Biochem.* 64, 381-387.
- [6] Dixit, S.N., Waring, A.J. and Vanderkooi, J.M. (1981) *FEBS Lett.* 125, 86-88.
- [7] Horie, T. and Vanderkooi, J.M. (1981) *Biochim. Biophys. Acta* 670, 294-297.
- [8] McLendon, G.L., Winkler, J.R., Mocera, D.G., Mauk, M.R., Mauk, A.G. and Gray, H.B. (1984), in press.
- [9] McGourty, J.L., Blough, N.V. and Hoffman, B.M. (1983) *J. Am. Chem. Soc.* 105, 4470-4472.