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AN EXTRINSIC ROOM TEMPERATURE PHOSPHORESCENT PROBE. REMARKABLE SHIELDING OF BENZOPHENONE TRIPLET AT THE ACTIVE SITE OF CHYMOTRYPSIN

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SUMMARY: Benzophenone can be used as an extrinsic triplet state probe, as its phosphorescence, a broad band centered at 445 nm, is readily observable in aqueous solution at room temperature. When bound covalently as an acyl enzyme at the active site of chymotrypsin, the benzophenone probe produces phosphorescence which is unusually resistant to quenching by O_2, trans-cinnamic acid, and H_3O^+. Sodium 2-naphthalenesulfonate quenches the phosphorescence, probably indirectly. The quenching data indicate that the local protein structure at the enzyme active site provides a rigid and protective substrate environment, which is not penetrated by even the smallest triplet quenchers.

Luminescence, produced either by intrinsic emitters such as tryptophan or by extrinsically attached probes, has become a standard tool in the study of the structure and dynamics of macromolecules. Although the bulk of work has employed fluorescence techniques (1-6), it is clear that the longer lived triplet state can complement the information available from fluorescence measurements in several important ways. These include greater sensitivity in measuring inaccessability of buried regions (7,8), determination of longer rotational correlation times characteristic of supramolecular assemblies or of proteins embedded in membranes (9,10), and measurement of relatively short interprobe distances using triplet-triplet energy transfer (11-15).

Despite widely quoted assertions to the contrary, intrinsic phosphorescence from tryptophan residues of many (7,8,16-19) but not all (7) proteins remains observable at temperatures as high as 40 C. In contrast extrinsic triplet state probes have been studied only by triplet-triplet absorption (9,10,20,21).

We report here that the benzophenone group, bound either covalently or as a Michaelis complex at the active site of chymotrypsin, produces phosphores-

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cence of high intensity, readily observable in aqueous solution at room temperature. The remarkable protection of this extrinsic phosphorescent probe from a variety of external quenchers raises interesting questions about the conformational flexibility of globular proteins, particularly at their binding sites, as well as about the use of luminescence quenching as a technique for measurement of protein dynamics.

MATERIALS AND METHODS

The sources or preparative methods for CT[1], NP-BPA, BPAA, and BPACT have been described previously (22). Benzophenone was recrystallized from ethanol and water. NS was purified by three recrystallizations from ethanol and water. Other materials were of the highest grade commercially available and were used without further purification. All solutions were prepared using triply distilled water, the second distillation being from acidic potassium permanganate.

Excitation and emission spectra were recorded on a Perkin-Elmer Hitachi MPK-2A spectrophotofluorimeter at 25 C. Intrinsic protein emission interfered at wavelengths below 400 nm. Solutions were placed in a 1 x 1 x 4 cm quartz cell, from which oxygen could be removed by passage of a nitrogen stream for at least 15 min.

Photoinactivation reactions were carried out as previously described (22).

RESULTS

BPACT, a chymotrypsin (E.C.3.4.21.1) derivative containing the benzophenone group covalently attached to ser 195, was prepared by acylation of the corresponding p-nitrophenyl ester. This acyl enzyme is stable at low pH, where deacylation is precluded by protonation of the essential histidine of CT. Figure 1 shows the room temperature excitation and total emission spectra of BPACT and for comparison emission spectra of BPAA and of benzophenone itself. All of the emission spectra likely combine the major phosphorescence band, centered at 440-445 nm, with a minor component due to activation controlled delayed fluorescence, near the blue end of the spectrum, as has been demonstrated in the room temperature emission of benzophenone (23,24).

Because BPACT undergoes deacylation at neutral pH, its emission spectrum cannot be reliably obtained under such conditions. On the other hand, emission

[1] Abbreviations: CT, α-chymotrypsin; BPAA, p-benzoylphenylacetic acid; NP-BPA, p-nitrophenyl p-benzoylphenylacetate; BPACT, p-benzoylphenylacetyl-α-chymotrypsin; NS, sodium 2-naphthalenesulfonate.

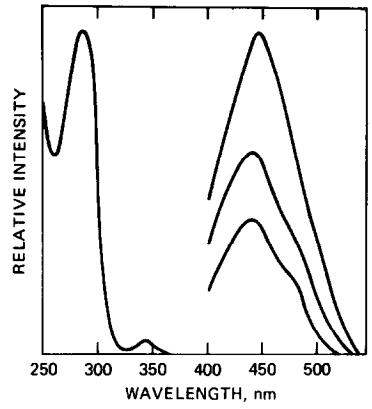

Figure 1. Room temperature excitation (left) and emission (right, upper curve) spectra of BPACT at pH 2.0. Room temperature emission spectra of BPAA (middle) and benzophenone (lower) at pH 5.5 are also shown. Intensities are normalized for equivalent numbers of photons absorbed. Actual concentrations range from 10-274 μM. CT itself does not emit at comparable wavelengths. Excitation wavelength for emission spectra is 366 nm; for the excitation spectrum emission is monitored at 425 nm.

spectra for the small molecules can be obtained only at pH 4 or higher, since phosphorescence of these derivatives is quenched in acid media (table 1, see also reference 25).

Phosphorescence is also observed when the benzophenone group is bound noncovalently at the CT active site. This is demonstrated by the emission produced by a mixture of CT and NP-BPA at pH 2, under which conditions a substantial fraction of the ester is bound as Michaelis complex, but its rate of enzymic hydrolysis is negligible. Phosphorescence cannot be due to free ester, since this is quenched by acid (table 1). That the ester is bound at the active site has been previously demonstrated by inhibition kinetics (22).

All phosphorescence of the protein bound probes disappears when the protein structure is unfolded by treatment with detergent.

Phosphorescence quenching data for the triplet states of benzophenone, BPAA, and the protein bound derivatives are shown in table 1. The surprising result is that the covalently labeled protein, BPACT, is totally unquenched by O_2, trans-cinnamic acid, or H_3O^+, all of which efficiently quench the phosphorescence of the small molecules. NS, however, does quench phosphorescence of BPACT as well as that of benzophenone. Stern-Volmer plots of these data appear in figure 2.

Table 1. Quenching of free and bound benzophenone derivatives.[a]

Phosphorophore	Quencher		pH	Phosphorescence intensity[b]
	Identity	Concentration, μM		
CT (control)			5.5	<1
CT (control)			2.0	<1
Benzophenone			5.5	59.6
Benzophenone	H_3O^+	10^4	2.0	<1
BPAA			5.5	90.4
BPAA	H_3O^+	10^4	2.0	<1
BPAA	O_2	280[c]	5.5	<1
BPACT	H_3O^+	10^4	2.0	143
BPACT	O_2	280[c]	2.0	143
BPACT	tCA[d]	880	2.0	145
NP-BPA	H_3O^+	10^4	2.0	<1
NP-BPA·CT[e]	H_3O^+	10^4	2.0	11.8
Benzophenone	NS	10.0[f]	5.5	19.0
BPACT	NS	10.0[f]	2.0	109

[a]exciting wavelength 366 nm; emission monitored at 425 nm. [b]relative values, normalized for equivalent number of photons absorbed. [c]air saturated. [d]trans-cinnamic acid. [e]noncovalent complex produced by mixing 28.9 μM CT and 216 μM NP-BPA. 72% of the enzyme active sites are occupied under these conditions. [f]see figure 2 for variation of intensity with NS concentration.

Independent confirmation of the efficiencies (or lack thereof) of triplet state quenchers lies in the effect of these quenchers on the photoreaction of BPACT, which can be monitored in several ways, including most simply the loss

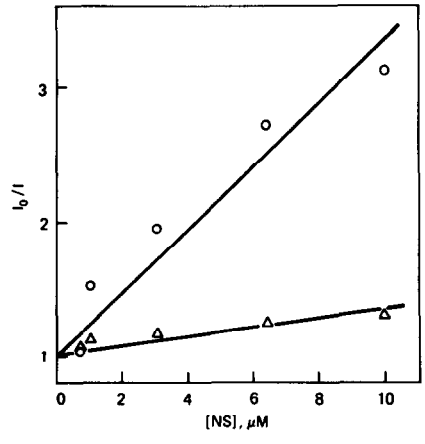

Figure 2. Stern-Volmer plots of quenching of phosphorescence emission from benzophenone (o) and BPACT (Δ) by NS. Excitation wavelength is 366 nm; emission is monitored at 425 nm. Calculated values of $k_q\tau$ from least squares analyses of the slopes are (2.4 ± 0.4) x 10^5 M^{-1} and (3.0 ± 0.4) x 10^4 M^{-1} respectively.

of enzymic activity (22). The rate of photoinactivation is totally insensitive to any of the quenchers studied, except NS, which at a concentration of 58 μM approximately halves the rate, compared to the control.

DISCUSSION

Benzophenone is the first extrinsic phosphorescent probe for general use at room temperature in aqueous solution. Its n,π^* state is produced from excitation at 340-365 nm, a region clear of protein absorption. Emission in the 430-450 nm range is easily distinguishable from normal protein fluorescence. Although nonluminescent triplet state probes can be studied by triplet-triplet absorption, the emission of a phosphorescent group allows unambiguous identification of the excited state and offers the further advantage of steady state observation on conventional instruments as well as the possibility of flash studies.

The benzophenone probe in BPACT is totally protected from O_2 quenching in air saturated solutions. Taking the triplet lifetime as 100 μs,[2] the less than 5% (experimental error) quenching observed at $[O_2] = 2.8 \times 10^{-4}$ M (air saturated at 25 C) implies $k_q < 1.9 \times 10^6\ M^{-1}s^{-1}$, where k_q is the bimolecular rate constant for quenching. In other words, quenching is slowed from a diffusion controlled rate by over three orders of magnitude.

Similarly the absence of observable quenching by trans-cinnamic acid at concentrations up to 0.88 mM leads to an upper limit of k_q of $6 \times 10^5\ M^{-1}s^{-1}$. The failure of H_3O^+ to quench at pH 2 implies a difference in quenching rates of at least 2000 between free and bound benzophenone groups, although the mechanism of proton quenching is unclear (25).

The protection against quenching is surprising in view of the fact that O_2 quenches the intrinsic tryptophan fluorescence of the same protein, including that from two deeply buried residues (26), with a rate constant of $2 \times 10^9\ M^{-1}s^{-1}$, or nearly diffusion controlled (27).

[2]Estimate based on published values of 65 μs (23) and 200 μs (25) for free benzophenone in water at room temperature.

The nature of the conformational flexibility that allows quenching of buried excited states remains unresolved, despite extensive study (28). One model postulates diffusion of quencher through the core of the protein as a result of small but random fluctuations in structure (27,29,30), while in an alternative approach, a concerted transition between open and closed forms governs the rate of quenching (7,8). The latter model is supported primarily by studies of intrinsic phosphorescence of liver alcohol dehydrogenase, the structure of which imposes a very large barrier to oxygen quenching. The present data, which also show severe restrictions on oxygen quenching, are certainly in better accord with this concerted model.

It is possible, however, that both models are correct, but for different regions of protein structure. It is notable that in the three instances of severely restricted quenching (table 2), emission is produced at or near a specific binding site. In alcohol dehydrogenase, the phosphorescence is produced from a tryptophan located in the NAD binding domain (31), not far from solvent. In the present work, as well as in G actin (32), emission is produced at the active site itself. These data suggest that the active site regions are

Table 2. Quenching constants for a variety of singlet and triplet state probes of macromolecules.

	Probe	Monitoring method	Macromolecule	Quencher	Quenching rate constant, $M^{-1}s^{-1}$	Reference
singlets	tryptophan (intrinsic)	fluorescence	14 proteins	O_2	2-7 x 10^9	27
	pyrene dihydrodiol epoxide	fluorescence	DNA	O_2	9 x 10^9	35
	tryptophan (intrinsic)	fluorescence	7 proteins	acrylamide	0.05[a]-2 x 10^9	30
	dansyl	fluorescence	F and G actins	acrylamide	0.2-2 x 10^8	36
	etheno ATP	fluorescence	G actin	amino acids	<6 x 10^6	32
triplets	tryptophan (intrinsic)	phosphorescence	liver alcohol dehydrogenase	O_2	6.5 x 10^4	7
	benzophenone	phosphorescence	chymotrypsin	O_2	<1.9 x 10^6	this work
	acetone	phosphorescence	horseradish peroxidase	O_2	2 x 10^8	37
	tryptophan (intrinsic)	triplet-triplet absorption	human serum albumin	O_2	9 x 10^7	18
	eosin	triplet-triplet absorption	5 proteins	O_2	~3 x 10^8	10
	6 polynuclear aromatics	triplet-triplet absorption	DNA	O_2	1-2 x 10^8	21

[a] range limited by experimental methodology; in azurin no quenching was detectable.

a great deal more rigid than previously supposed, even while the remainder of the protein may remain flexible.

It is of interest that in free CT one of the tryptophans efficiently quenched by O_2 is trp 215, which forms part of the lining of the very cavity which binds the benzophenone in BPACT. Substrate binding may tighten the protein structure in the active site to prevent O_2 penetration, while in the free enzyme O_2 may readily diffuse through a more open structure.

There are a number of explanations for quenching by NS, but the simplest is that this "quencher" binds to a second hydrophobic site of CT, as observed for alkanoyl benzoates (33). The resulting perturbation of protein structure, sufficient to inhibit enzymic activity (33), opens the binding site enough to allow quenching by H_3O^+.

A disconcerting possibility remains, stemming from the fact that at least with O_2, quenching of singlets is indiscriminately efficient, whereas quenching of triplets is consistently slower, sometimes spectacularly so (table 2). It is possible that as yet unknown differences exist in requirements for singlet and triplet quenching which are quite independent of protein structure. This doubt is reinforced by the recent work of Turro (34) on phosphorescence from within micelles, in which unusual "protection" from O_2 was required to explain the data. Resolution of this problem should be possible by comparing quenching rates of identically placed singlet and triplet state probes, a project which is now being undertaken in these laboratories.

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