Phosphorescence/microwave double-resonance spectra of tryptophan perturbed by methylmercury(II)

RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE/OPTICAL DETECTION OF TRIPLET-STATE MAGNETIC RESONANCE/HEAVY ATOM EFFECT

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ABSTRACT Amplitude-modulated phosphorescence/microwave double-resonance (AM-PMDR) spectra are reported for complexes of methylmercury(II) cation, designated CH₃Hg⁺, with tryptophan and glyceraldehyde-3-phosphate dehydrogenase (GPDHase; from rabbit muscle). Wavelength shifts are observed in the AM-PMDR spectra of CH₃Hg⁺-tryptophan, which are obtained by microwave pumping in distinct zero-field D + E magnetic resonance transitions, demonstrating that AM-PMDR can be used to display selectively the phosphorescence spectra of structurally distinct complexes with different zero-field splittings. The AM-PMDR spectra accurately represent the phosphorescence of CH₃Hg⁺-tryptophan. Binding of CH₃Hg⁺ to a cysteine site of GPDHase perturbs the luminescence of one of the two optically resolved tryptophans. The AM-PMDR spectrum of the perturbed tryptophan is obtained by microwave pumping of the D + E magnetic resonance signal, which can be observed optically only in the presence of a heavy atom perturbation. The resulting spectrum is broadened and shifted to the blue relative to the corresponding tryptophan phosphorescence spectrum of the uncomplexed enzyme. Comparison of the AM-PMDR spectra of CH₃Hg⁺-tryptophan and CH₃Hg⁺-GPDHase suggests that there are differences in the mechanisms of heavy atom perturbation in these complexes.

Optical detection of triplet-state magnetic resonance (ODMR) has been used for several years for the investigation of biologically interesting molecules, including proteins and nucleic acids (1). In typical experiments, phosphorescence of the sample (cooled to below liquid helium temperature) is monitored while the zero-field magnetic resonance transitions are simultaneously irradiated with microwaves. Triplet sublevel population shifts induced by resonant microwaves generally result in an optical response because of differences in the kinetic and radiative properties between the sublevels. A rather neglected ODMR method as far as the application to biological molecules is concerned is amplitude-modulated phosphorescence/microwave double resonance (AM-PMDR) (2). In this method, a zero-field ODMR signal of the sample is first located by monitoring the total phosphorescence emission (or a selected part of it, such as the 0,0-band). The microwave frequency is then fixed at resonance, with amplitude modulation (AM) at frequency f₀. The detection wavelength, dispersed by a monochromator, is swept through the phosphorescence region, and the component at f₀ is selected by a coherent detector. AM-PMDR can be used to discriminate between competing chromophores if the microwave frequency is chosen to correspond to an ODMR signal of only one of them. In this way a component at f₀ will be present only in the luminescence of the molecule of interest.

We have found (5, 6) that the methylmercury(II) cation, designated CH₃Hg⁺, which binds to the α-nitrogen atom of tryptophan (7), leads to a heavy atom effect on the indole chromophore. Based on the crystal structure of the analogous tyrosine complex (8), which also exhibits a heavy atom effect (9), the structure is folded, bringing the Hg atom into contact with the π-electrons of the aromatic ring. The highest frequency (D + E) ODMR transition of tryptophan normally is not observed because both of the triplet sublevels involved in this transition decay radiationlessly. In the CH₃Hg⁺-tryptophan complex, on the other hand, the heavy atom effect induces radiative processes in these sublevels, and a D + E ODMR signal appears (5, 6). The region of the D + E signal is resolved into several peaks that vary between 4.23 and 6.8 GHz (6), indicating structural variations in the complexes.

CH₃Hg⁺ is known to bind tightly to accessible sulfhydryl residues in cysteine-containing proteins (10). The binding can be limited to sulfhydrys by introducing CH₃Hg⁺ as the iodide or bound to a sulfur-containing ligand such as mercaptoethanol (7). In a previous investigation (11), we reported a specific Hg⁺-tryptophan interaction in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GPDHase) when CH₃Hg⁺ was complexed with the accessible cysteines of the enzyme. Of the two optically-resolved tryptophan phosphorescence spectra of GPDHase, one remains unperturbed upon binding CH₃Hg⁺, whereas the second undergoes broadening and a drastic reduction of its phosphorescence lifetime. The appearance of a tryptophan D + E ODMR signal in the complexed enzyme is also diagnostic of the heavy atom effect. Because the spectrum of the unperturbed tryptophan is still present, it is difficult to deduce the underlying spectrum of the perturbed tryptophan.

In this communication, we report the resolved AM-PMDR spectra of CH₃Hg⁺-tryptophan, which are obtained by microwave pumping in distinct D + E transitions, and also the AM-PMDR spectrum of the perturbed tryptophan of rabbit GPDHase. This is a novel use of AM-PMDR to separate interfering spectra of triplet states in a biopolymer.

Abbreviations: GPDHase, glyceraldehyde-3-phosphate dehydrogenase; AM, amplitude modulation; AM-PMDR, amplitude-modulated phosphorescence/microwave double resonance; ODMR, optical detection of triplet-state magnetic resonance; FM, frequency modulation.

Although phosphorescence is not a resonance phenomenon, there is a strong analogy between the experiment described here and genuine double-resonance measurements such as electron/nuclear double resonance, designated ENDOR. The term PMDR was first used (3) to distinguish between ODMR, in which the entire phosphorescence was monitored, and an experiment in which the microwave responses of individual vibrational bands dispersed by a monochromator are observed. The term PMDR has since come into common usage to conveniently refer to experiments in which magnetic resonance effects are measured at various optical wavelengths (e.g., see ref. 4).

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MATERIALS AND METHODS

GPDHase from rabbit muscle was obtained from Sigma as a crystalline suspension in 2.6 M (NH₄)₂SO₄. It was centrifuged for about 20 min at 12,000 × g, and the pellet was dissolved in 0.01 M potassium phosphate buffer (pH 7.2) containing 1 mM mercaptoethanol. The solution was applied to a Pharmacia PD-10 desalting column and eluted with the same buffer. These operations were carried out between 0 and 5°C. The protein concentration was 25 mg/ml as determined by the absorbance at 276 nm (A₂₇₆ × 0.95 = mg/ml). Phosphorescence spectra of the uncomplexed enzyme were obtained from a portion of this solution diluted 5:4 (vol/vol) with ethylene glycol (Matheson, Coleman and Bell) and chloroform. The CH₃Hg(II)-GPDHase complex was prepared by diluting the aqueous sample 5:4 (vol/vol) with ethylene glycol containing 27 mM CH₃HgAl (Alfa, Danvers, MA, 98%). The iodide was used to limit complexing to enzymesulfhydryl groups. L-tryptophan (Calbiochem) was made 0.5 mM in 10 mM phosphate buffer, pH 7/ethylene glycol 1:1 (vol/vol). The CH₃Hg(II)-tryptophan complex was formed by adding a 5-fold molar excess of CH₃HgOH (Alfa) to this solution.²

Phosphorescence and AM-PMDR measurements were made by using the apparatus as described (11). Phosphorescence spectra were recorded at 4.2 K, whereas AM-PMDR measurements were made at the pumped He temperature, 1.1–1.2 K. For the phosphorescence measurements, we utilized a rotating can shutter with a dead time of 0.8 msec, whereas AM-PMDR was modulated at 2–3 kHz over the inhomogeneously broadened ODMR transition to improve the sensitivity. The microwave amplitude was modulated at a lower audio frequency by using a PIN diode modulator. The output of the audio oscillator also was used as a reference signal to drive an Ortec model 9412A phase-sensitive detector. The intensity of the AM-PMDR spectrum was found to vary with the AM frequency in agreement with previous observations (12). The microwaves were amplified to an average power of 500 mW before being applied to the sample through a coaxial line inserted into the Dewar flask. The sample, contained in a 1-mm Suprasil quartz tube, was inserted into a copper helical slow-wave structure that terminated the line. The sample was optically pumped at 300 nm (16-nm band-pass) using the filtered output of a 100-W high-pressure Hg arc. The luminescence was passed through a McPherson model 2051 1-m monochromator with 2.5-nm slits and was detected with a cooled EMI model 9789QA photomultiplier. The photocurrent was amplified by a Pacific Photometrics model 124 photometer having a risetime of 150 μsec and subsequently was fed to the phase-sensitive detector. The output signal was filtered and finally sent to a Nicolet model 1072 signal averager. For signal averaging, the monochromator was swept through the tryptophan phosphorescence region at 20 nm/min (except as noted) by using a GCA/McPherson model 786 digital scan controller that was calibrated with a low-pressure Hg lamp. Output pulses from the scan controller were also used as a trigger source to advance the signal averager channels synchronously with the wavelength scan. At the end of each complete wavelength scan, a home-built retrowatcher returned the monochromator to the starting wavelength in preparation for the next one. Phosphorescence spectra were not signal-averaged, but a single scan was stored in the signal averager by coupling the output of the photometer amplifier directly to the signal averager through a 1-sec resistance-capacitance filter.

RESULTS

The phosphorescence spectra of tryptophan and the complex CH₃Hg(II)-tryptophan are shown in Fig. 1 (spectra A and B, respectively). Under the conditions of sample preparation, the tryptophan is essentially all in the form of the complex (5, 6); thus, the contribution of tryptophan to spectrum B is negligible. Complex formation with CH₃Hg(II) was accompanied by a red shift of the 0.0-band peak from 405.7 to 411.3 nm. In addition, the heavy atom enhancement of the phosphorescence intensity occurred selectively in the regions of the prominent bands at 411.3 and 437.8 nm in the complex. We have noted (6) that the CH₃Hg(II)-tryptophan complexes are heterogeneous in that a distribution of phosphorescence lifetimes is observed at 77 K as well as resolved ODMR peaks in the D + E signal region. The higher frequency ODMR signals were found to be associated with shorter triplet-state lifetimes in transient measurements. We obtained the AM-PMDR spectra of CH₃Hg(II)-tryptophan complexes associated with both the lowest frequency (4.23 GHz) and with the highest frequency (6.6 GHz) D + E transitions (Fig. 1, spectra C and D, respectively). Both spectra resemble the phosphorescence spectrum of CH₃Hg(II)-tryptophan closely. The 0.0-band peaks of the AM-PMDR spectra are each shifted relative to the 0.0-peak of the phosphorescence, however. The 0.0-band of the AM-PMDR spectrum associated with the 4.23-GHz transition is shifted to the red to 412.4 nm, whereas that associated with the 6.6-GHz transition is shifted to the blue to 410.3 nm. We found that lower AM frequencies were required to observe AM-PMDR spectra at 4.23 GHz than were required at 6.6 GHz. The latter transition is associated with the shorter-lived complexes whose sublevel populations can respond to microwave modulation at a higher rate. We were concerned initially that the observed spectra might be caused by spurious modulation of the detected luminescence, which could originate, for instance, from fluctuations in the optical properties of the superfluid He caused by modulation of the microwave power. This possibility can be discounted, however, because the AM-PMDR spectrum observed with different microwave frequencies were clearly distinguishable from each other and also from the phosphorescence. We also found that when the microwave frequency was moved out of the D + E signal region, no AM-PMDR spectrum was observed under otherwise identical conditions.

The phosphorescence spectrum of rabbit muscle GPDHase is given in Fig. 2, spectrum A. It is characterized by relatively narrow, well-resolved bands. Well-resolved phosphorescence spectra are expected from tryptophan sites that are buried in the enzyme structure and, therefore, are not subject to heterogeneous broadening that accompanies interactions with the solvent (13, 14). Specifically, two complete spectra are superimposed that have 0,0-bands at 405.5 and 415.7 nm (indicated by vertical arrows in Fig. 2, spectrum A, labeled 1 and 2, respectively). We have reported the distinct ODMR spectra as

²Organomerscurials are extremely toxic. Care was taken to avoid inhalation, or contact with the skin. The CH₃Hg(II) compounds, waste solutions, and solid waste were kept isolated in a separate, dedicated hood in which all sample preparation was carried out.

³We refer to the ODMR transitions that occur above 4 GHz as D + E transitions mainly for convenience. In unperturbed tryptophan, the D and E bands are about 4.2 GHz apart, and the D + E transition is about 4.2 GHz and occurs between T₁ and T₂ (perpendicular to the indole plane). The D + E and 2E transitions occur at about 1.7 and 2.5 GHz, respectively. It is possible that the 6.6-GHz transition of CH₃Hg(II)-tryptophan, in particular, may not occur between T₁ and T₂ because of the large and at present unknown zero-field-splitting shifts induced by the Hg atom in this complex. The sublevel assignment of the ODMR lines at 2.23 GHz and 6.6 GHz are not relevant to the arguments presented, however, as long as they occur in different complexes.
sociated with tryptophan 1 and tryptophan 2 previously (11). No D + E signal was observed when monitoring either of these 0,0-bands.

The spectrum of the CH$_3$Hg(II)−GPDHase complex is given in Fig. 2 (spectrum B). The most obvious difference between this and the unperturbed GPDHase phosphorescence spectrum is the absence of the resolved bands of tryptophan 2. All of the remaining resolved bands can be assigned to tryptophan 1 by comparison with the highly resolved phosphorescence spectrum of the single tryptophan of ribonuclease T1 (14). The presence of short-lived components in the phosphorescence spectrum of CH$_3$Hg(II)−GPDHase was attributed to the selective heavy atom perturbation of tryptophan 2, and the absence of resolved bands in the spectrum was taken as evidence that the perturbation of tryptophan 2 is accompanied by considerable spectral broadening (11). Additional evidence that the short-lived phosphorescence components originate from perturbed tryptophan was the observation of a new ODMR signal at 4.5 GHz, which is in the D + E signal region of CH$_3$Hg(II)−tryptophan. When the 4.5-GHz transition was driven in an AM-PMDR experiment, spectrum C in Fig. 2 was observed. The sharp phosphorescence peaks and the fluorescence of tryptophan 1 are absent from this spectrum, which is not, therefore, the result of spurious modulation of the total luminescence. We assign this spectrum as the AM-PMDR of tryptophan 2 perturbed by sulhydryl binding of CH$_3$Hg(II). We suggest that it represents the phosphorescence of tryptophan 2 in the perturbed enzyme, which thereby is confirmed to be broad and relatively structureless.

FIG. 1. Phosphorescence spectra of tryptophan (spectrum A) and CH$_3$Hg(II)−tryptophan (spectrum B); AM-PMDR spectra of CH$_3$Hg(II)−tryptophan at 4.29 GHz (spectrum C) and at 6.8 GHz (spectrum D). For spectrum C, frequency modulation (FM) limits were 3.9 and 4.3 GHz and the AM frequency was 17 Hz. The spectrum is the result of 13 scans. For spectrum D, FM limits were 6.35 and 6.85 GHz, the AM frequency was 230 Hz, and the scan rate was 2 nm/min. The spectrum is the result of three scans.

FIG. 2. Phosphorescence spectra of rabbit muscle GPDHase (spectrum A) and CH$_3$Hg(II)−GPDHase (spectrum B) and the AM-PMDR spectrum of CH$_3$Hg(II)−GPDHase (spectrum C). For spectrum C, FM limits were 3.9 and 5.0 GHz, and the AM frequency was 20 Hz. The spectrum is the result of 83 scans. The arrows in spectrum A indicate the optically resolved 0,0-bands of tryptophans 1 and 2.

**DISCUSSION**

The measurements reported on CH$_3$Hg(II)−tryptophan show that the AM-PMDR spectra are a good representation of the phosphorescence. Furthermore, the wavelength shifts of the AM-PMDR spectra, which are associated with structurally distinct complexes with different zero-field splittings and triplet-state lifetimes, demonstrates that AM-PMDR can be used to selectively display the phosphorescence spectra of the components. It has been noted (14–16) that phosphorescence wavelength shifts can be correlated with the zero-field-splitting parameters D − E of tryptophan in different environments. Theoretical explanations of this effect have been offered (17–20). The mechanisms are based on electronic energy-level shifts which arise from the electric field of the solvent environment. The Stark shifts of the triplet state and of the electronic states that are coupled with its spin sublevels through spin−orbit coupling result in shifts in the zero-field splitting as a consequence of changes in the energy denominator in the second-order energy perturbation expression. The common Stark effect origin of shifts in zero-field splitting and phosphorescence wavelength has been confirmed experimentally by using externally applied electric fields (21). The important feature of this mechanism is that the solvent environment has no influence on the spin−orbit coupling mechanism itself or on its magnitude. In the case of CH$_3$Hg(II)−tryptophan complexing, the situation is more complicated in that the solvent environment contains the Hg atom, which affects the spin−orbit coupling of tryptophan by an external heavy atom effect. Thus, a distribution of complex configurations would be expected to exhibit a distribution of spin−orbit coupling strengths. Variations in the
strength of spin–orbit coupling will be manifested mainly in the zero-field splitting and in the triplet-state lifetime but not significantly in the phosphorescence wavelength, in comparison with the solvent Stark shifts.

For tryptophan itself, which is subject only to solvent-induced Stark effects, $D + E$ is found to be relatively invariant in a number of distinct solvent environments. The value of $D + E$ for 19 distinct tryptophan environments in various peptides and enzymes varies only between 4.01 and 4.40 GHz; the average is 4.23 GHz ($\sigma = 0.09$). The 0,0-band maxima of this set vary between 404 and 416 nm with no apparent correlation between $D + E$ and the 0,0-band wavelength (11, 14). In the CH$_3$Hg(II)–tryptophan complexes, the $D + E$ region is wide and structured, with peaks ranging between 4.23 and 6.6 GHz. This large a range of zero-field splitting is unlikely to result from Stark effects; it is probably the result of variation in spin–orbit coupling due to the proximity of the heavy atom, as discussed above. This interpretation is supported by the observation that the triplet-state lifetime of the complex with the “normal” $D + E$ equal to 10 msec, whereas that of the complex with $D + E$ equal to 6.6 GHz is reduced to 1 msec (6). On the other hand, there is relatively little dispersion in the 0,0-band maxima (ca. 2.1 nm), which suggests that Stark effect variations among the complexes are relatively small.

In earlier work (11) we suggested, arguing by analogy with the x-ray structure of lobster GDPHase (22), that the heavy atom perturbation in rabbit GDPHase arises from a specific interaction between tryptophan-310 and CH$_3$Hg(II) complexed with cysteine-281. Recent measurements in this laboratory (unpublished data) show that yeast GDPHase, which contains serine substituted for cysteine-281, does not show a tryptophan heavy atom effect when complexed with CH$_3$Hg(II). This observation adds support to the assignment of the interaction that leads to the heavy atom effect in the rabbit enzyme. Based on the x-ray crystal structures of GDPHase from lobster (22) and Bacillus stearothermophilus (23), tryptophan-310 is a buried residue whose N–H is internally hydrogen bonded with aspartic acid-293. These residues are conserved in all the GDPHase that have been completely sequenced to date [lobster (24), yeast (25), pig (26), B. stearothermophilus (23), and Thermus aquaticus (27)]. The AM-PMDR spectrum of the complexed rabbit enzyme (Fig. 2, spectrum C) is quite different in appearance from that of the model complexes, CH$_3$Hg(II)–tryptophan. The spectrum is significantly shifted to the blue relative to the phosphorescence of tryptophan 2 in the complexed enzyme. Such a shift is expected as a result of the disruption of local interactions, which must accompany the binding of CH$_3$Hg(II) in the vicinity of tryptophan 2. The spectrum has relatively steep onsets near 408 and 436 nm, but it is not characterized by the prominent peaks that are shown by the CH$_3$Hg(II)–tryptophan complexes. There appears to be an enhancement of the intensity in low-frequency vibronic bands of the spectrum. Under conditions of limited resolution, this could well lead to a spectrum in which the region between the prominent tryptophan vibronic peaks (0,0 and ca. 0,0–1500 cm$^{-1}$) is filled in to give a plateau-like appearance. It has been observed (4) that in $(\pi, \pi^*)$-triplet states of halogen-substituted aromatic molecules, such as $p$-dibromobenzene, $p$-chloroaniline, and 2,3-dichloroquinoxaline, a significant mechanism for the radiative heavy atom enhancement is second-order vibronic spin–orbit coupling (28) that uses low-frequency halogen out-of-plane bending modes. Although ours is obviously a very different system, an analogous mechanism may be operative in the CH$_3$Hg(II)–GDPHase complex if Hg atom motions are coupled with low-frequency modes of the indole structure. It is apparent, in any event, that different heavy-atom-effect mechanisms are operative in the CH$_3$Hg(II)–tryptophan complex and in the CH$_3$Hg(II)–GDPHase complex.

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