Resonance Coherent Anti-Stokes Raman Scattering Spectra of Oxidized and Semiquinone Forms of *Clostridium MP* Flavodoxin†

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ABSTRACT: CARS (coherent anti-Stokes Raman scattering) spectra have been obtained for aqueous solutions of flavodoxin from *Clostridium MP* in oxidized and semiquinone forms by using excitation in the longer wavelength absorption bands. The oxidized spectrum is similar to previously obtained flavin spectra. Modest shifts in three of the bands, relative to aqueous flavin mononucleotide (FMN), reflect protein interactions at the binding site; the lack of shift of the 1257-cm^{-1} band indicates that the N_3H H bond to Glu-59 is comparable to that of FMN with H_2O. The semiquinone spectrum is the first to be obtained on this form of flavin. It differs markedly from that of oxidized flavin and is therefore a good fingerprint for semiquinone, readily distinguishable from the spectra expected for charge-transfer complexes, which can give similar long-wavelength absorptions. The semiquinone spectrum contains two prominent bands assignable to the pyrazinium portion of the molecule. Both oxidized and semiquinone forms of flavin show resonance enhancement of only one CARS band, the pyrazine breathing mode at 1584 and 1616 cm^{-1}, respectively, upon excitation in the shorter wavelength absorption bands, in contrast to the many CARS bands enhanced in the longer wavelength absorption bands. This parallelism supports the assignment of the two bands in each form to the same pair of π–π^* transitions, which are red shifted in the semiquinone due to the partial occupancy of the π^* orbital.

Recent studies using resonance Raman spectroscopy have deomonstrated the feasibility of monitoring flavin structure. The formidable interference from flavin fluorescence can be eliminated with the CARS[1] (coherent anti-Stokes Raman scattering) technique (Dutta et al., 1977). This is a form of nonlinear spectroscopy in which two phase-matched laser beams are used to generate the Raman signal as a coherent beam of light, which can be filtered spatially from the isotropic fluoresence (Begley et al., 1974). In some cases protein binding may quench the flavin fluorescence sufficiently to permit the acquisition of spontaneous Raman spectra, as Nishina et al. (1978) have shown for riboflavin-binding protein.

Flavodoxins offer a unique opportunity for exploring the systematics of flavin Raman spectroscopy, being the simplest (smallest) flavoproteins for which high-resolution X-ray structures are available (Watenpaugh et al., 1972; Burnett et al., 1974). Oxidized, semiquinone, and reduced forms of flavodoxin from *Clostridium MP* have been analyzed (Mayhew & Ludwig, 1975; Ludwig et al., 1976; Smith et al., 1977), and the likely interactions of the bound FMN with the protein have been established. We have obtained resonance CARS spectra of flavodoxin from *Clostridium MP* in both oxidized and semiquinone forms, and we offer a preliminary interpretation. This is the first report of flavin semiquinone Raman spectra.

Experimental Procedures

Clostridium MP flavodoxin was a gift from Professor M. Ludwig. Resonance CARS experiments were carried out with instrumentation described previously (Nestor, 1978). It consists of a Molectron N_2 laser pumping two dye lasers (ω_1 and ω_2). The outputs of the dye lasers are focused into the sample at an angle of $\sim 1°$, and the anti-Stokes output is monitored as one of the dye lasers (ω_2) is scanned relative to the fixed dye laser at ω_1. One millimolar solutions of flavodoxin were used for both oxidized and semiquinone forms. Flavodoxin semiquinone was prepared by anaerobic light irradiation in the presence of 0.08 M EDTA (Massey & Palmer, 1966), the absorption spectra being monitored before and after CARS spectroscopy. The 992-cm^{-1} CARS band of benzene was used to calibrate the spectrometer prior to the recording of flavin spectra, whose frequencies were determined from the peak shifts. N^5-Ethylflavin mononucleotide was prepared as described by Kemal et al. (1977) using FMN, CH_3CHO, and ninhydrin as the oxidizing agent. The concentration was ~ 1 mM. Fresh samples were prepared just before the experiments since the radical decomposed with time.

Results and Discussion

Flavin–Protein Interactions. Figure 1a shows the isoalloxazine structure and the ring numbering system. Aside from van der Waals contacts, the isoalloxazine ring–protein interactions in oxidized flavodoxin are limited to hydrogen bonds involving ring III, the pyrimidine ring (Burnett et al., 1974; Smith et al., 1977). O_2 and O_4 accept H bonds from peptide amide groups, while N_3H donates a H bond to a carboxylate O atom of Glu-59. In the semiquinone form there is an additional H bond between the N_5H group of the pyrazinium ring (see Figure 1a) and the carbonyl O atom of Gly-57. This interaction is believed to stabilize the neutral (blue) form of the semiquinone, raising the pK of the transition to the anionic (red) form by three units relative to the protein-free radical (Smith et al., 1977). In addition to the H bonds, there is a possible stacking interaction with the indole ring of Trp-90 (Smith et al., 1977).

Oxidized Flavodoxin. Figure 2 shows the CARS spectrum of oxidized flavodoxin, with the primary laser, ω_1, adjusted to 480 nm, in resonance with the origin of the first π–π^* isoalloxazine electronic transition (Eaton et al., 1975). At this wavelength the CARS bands show minimum distortion from Lorentzian line shapes (Dutta & Spiro, 1978). The flavodoxin

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[1] Abbreviations used: CARS, coherent anti-Stokes Raman scattering; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid.

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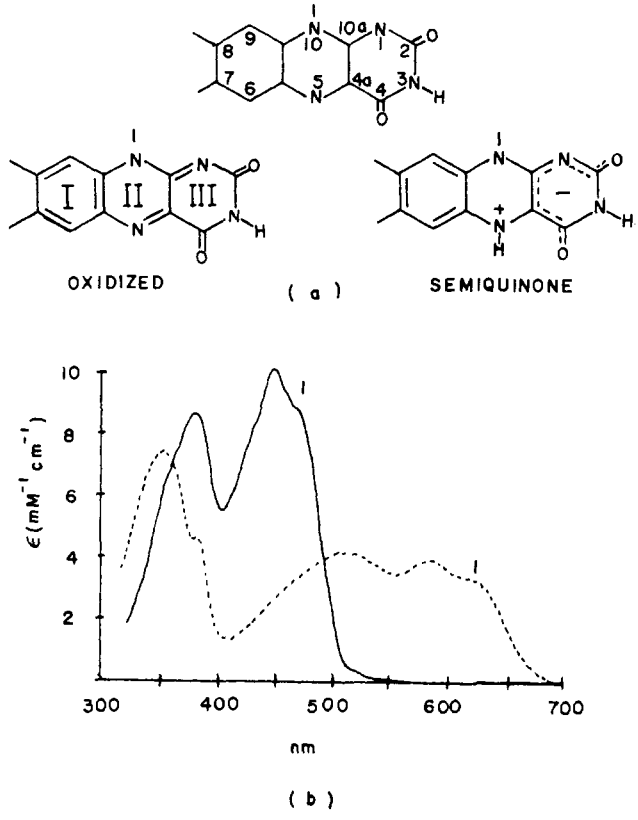
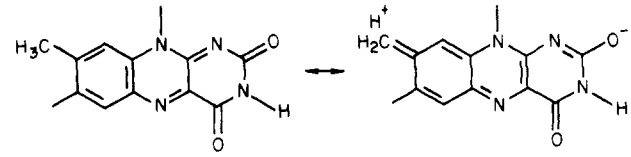


FIGURE 1: (a) Flavin ring numbering system and the structures of oxidized flavin and neutral semiquinone. (b) Absorption spectrum of oxidized (—) and neutral semiquinone (- - -) forms of *Clostridium MP* flavodoxin.

spectrum is of unusually good quality, and it is the best example of a flavin CARS spectrum that we have obtained. The features are typical of other flavoproteins (Dutta et al., 1977, 1978), except that the 1279-cm^{-1} band is unusually prominent. The strong bands at 1628, 1577, and 1409 cm^{-1} are all 7 cm^{-1} lower than they are in protein-free FMN. Similar shifts have been observed for glucose oxidase and riboflavin-binding protein (Dutta et al., 1977, 1978).

Tentative assignments of the oxidized flavin Raman bands have been inferred from frequency shifts upon isotope or chemical substitutions (Dutta et al., 1978, 1980; Nishina et al., 1978; Kitagawa et al., 1979a). The 1628-cm^{-1} band is localized mainly on ring I, as evidenced by its large shift in 7,8-dichlororiboflavin (Nishina et al., 1978). The 1577-cm^{-1} mode corresponds to a ring-breathing mode of pyrazine and is assignable to ring II (Kitagawa et al., 1979a; Dutta et al., 1980), while the 1409-cm^{-1} band is assignable to ring III, on the basis of its sensitivity to ring III substitutions (Dutta et al., 1980). There are several plausible contributors to the 7-cm^{-1} lowerings of these modes relative to those of aqueous FMN. H bonding to the heteroatoms of ring III is one possibility. Another is the likely destabilization of zwitterionic resonance forms of the type



in the hydrophobic environment of the flavin binding site. Such forms account for the appreciable 8-CH_3 H–D exchange rate in D_2O (Bullock & Jardetzky, 1965). The 1628-cm^{-1} mode corresponds to the 1607-cm^{-1} mode of *o*-xylene, which shifts to higher frequency when electron-donating substituents replace the methyl groups. The increase of the 1628-cm^{-1} frequency in aqueous FMN might therefore reflect the stabilization of the zwitterionic form in water relative to flavodoxin. Finally, the possible stacking interaction with Trp-90 might lower the frequencies to the extent that it resulted in partial electron transfer to flavin.

The H bonding at N_3 is expected to influence the frequency of the 1257-cm^{-1} mode in particular. This band disappears on N_3H deprotonation and shifts up by 30 cm^{-1} on N_3H deuteration (Dutta et al., 1978, 1980), presumably due to coupling with the N_3–H and N_3–D bending modes (Nishina et al., 1978). Its frequency is 10 cm^{-1} lower in riboflavin-binding protein than in aqueous flavin, and it disappears altogether in glucose oxidase (Dutta et al., 1978). The lack of a significant shift in flavodoxin (1257 vs. 1260 cm^{-1}) suggests that the strength of the H bond from N_3H to Glu-59 is about the same as to water for aqueous FMN. It is interesting that the plane of the Glu-59 carboxylate is perpendicular to that of the isoalloxazine ring (M. Ludwig, personal communication) so that the H-bond geometry is unfavorable.

Flavodoxin Semiquinone. Figure 3 shows the CARS spectrum of flavodoxin semiquinone in resonance with the longest wavelength absorption band. ω_1 was adjusted to the electronic origin at 620 nm (Eaton et al., 1975) in order to produce Lorentzian line shapes. The spectrum is very different from that of oxidized flavin and can easily serve as a fingerprint for semiquinone. This is encouraging, since the absorption spectrum (see Figure 1b) can sometimes be difficult to distinguish from those of charge-transfer complexes, which give long-wavelength absorptions of varying shapes and intensities (Massey & Ghisla, 1974; Williams, 1976). While such absorptions may provide resonance enhancement of Raman spectra, the frequencies of the bands are expected to be close to those of the individual partners in the charge-transfer complex (Kitagawa et al., 1979b). Thus the Raman spectrum should readily distinguish between the charge-transfer complex and semiquinone formation.

It will be difficult to compare the spectrum in Figure 3 with that of protein-free semiquinone. In the absence of protein interactions, disproportionation to oxidized and reduced forms is favored thermodynamically and is quite fast (Draper &

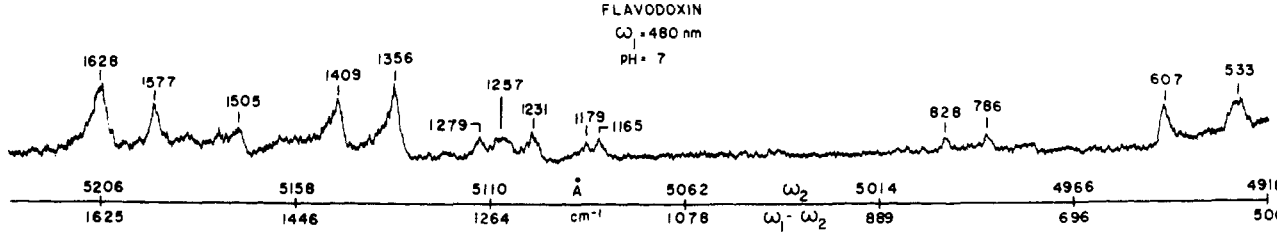


FIGURE 2: Resonance CARS spectrum of ~1 mM *Clostridium MP* flavodoxin (5 mM Tris, pH 7.3) at ω_1 = 480 nm. Laser pulse energies of ω_1 and ω_2 were 10 μJ at the sample; scan rate of ω_2 = 6 Å/min, 10 pulses/s; 30 pulses averaged.

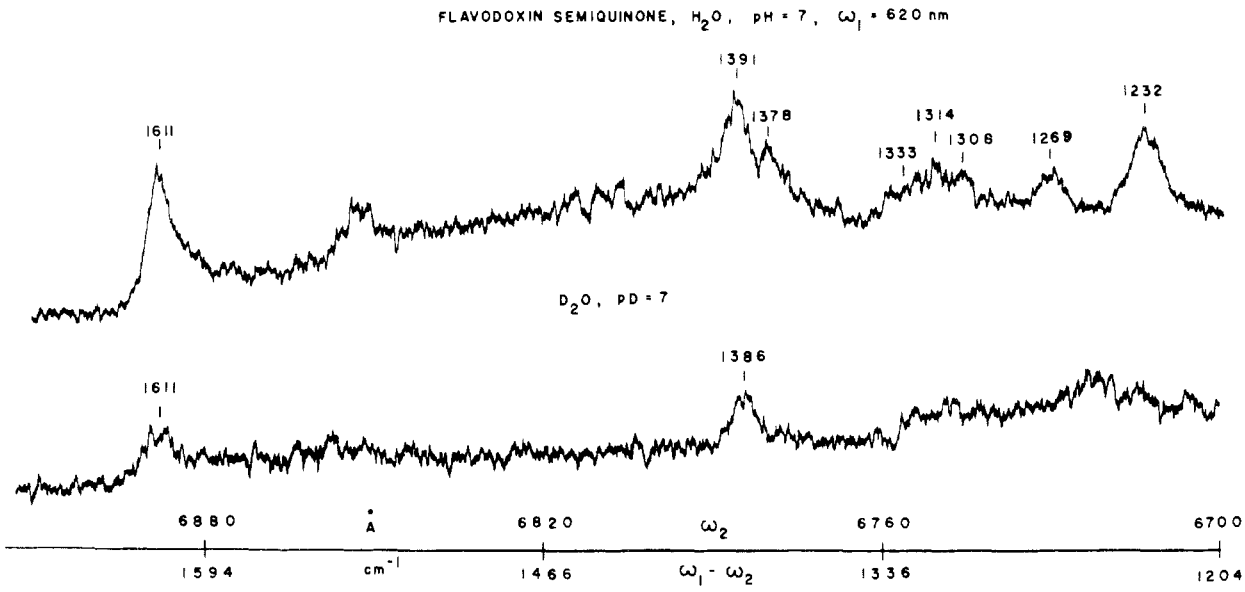


FIGURE 3: Resonance CARS spectrum of ~1 mM *Clostridium MP* flavodoxin semiquinone in H_2O and D_2O buffered with 5 mM phosphate at ω_1 = 620 nm. Same conditions as in Figure 2 except scan rate = 7.5 Å/min. Lower frequencies could not be obtained because of small phase-matching angles.

Ingraham, 1968). Hemmerich and Massey (Hemmerich et al., 1967; Walker et al., 1970) have shown that the flavin radical can be greatly stabilized by substitutions at N_5. We examined N^5-ethylflavin mononucleotide semiquinone (Kemal et al., 1977), but at the concentrations needed (2–3 mM) for CARS spectroscopy in resonance with the long-wavelength band, the radical decomposed during the course of the experiment. The shorter wavelength band was somewhat stronger (see Figure 1b), and we were able to obtain a CARS spectrum in resonance with this band (ω_1 = 520 nm) by using a lower concentration (~1 mM). Only one Raman band could be seen, however, which showed up quite strongly at 1616 cm^{-1}. This is strikingly reminiscent of the single strong band, at 1584 cm^{-1}, observed upon excitation in the short wavelength (370-nm) band of oxidized flavin (Nishimura & Tsuboi, 1978).

Both oxidized flavin and the neutral semiquinone therefore show similar resonance-enhancement patterns. Many modes are enhanced in resonance with the longer wavelength electronic transition, but only one is enhanced via the shorter wavelength one. Different excited-state geometries, resulting in different Frank–Condon overlaps, are implied for the two transitions. The parallelism in resonance enhancement between oxidized and semiquinone forms provides confirmation of the suggestion of Eaton et al. (1975), based on the polarized absorption spectra of flavodoxin crystals, that the absorption bands have the same orbital assignments, namely transitions from the two highest filled π orbitals to the lowest π^* orbital. The open shell structure accounts for the red-shifted energies of the semiquinone absorptions.

The fact that excitation in the shorter wavelength band selects the 1584-cm^{-1} band for enhancement in oxidized flavin and the 1616-cm^{-1} band in the model semiquinone implies that these two frequencies are associated with essentially the same normal mode, which *increases* by 32 cm^{-1} upon one-electron reduction of flavin. This reduction is accompanied by uptake of a proton at N_5 of the pyrazine ring, and it is striking that the 1584-cm^{-1} mode of pyrazine increases to 1618 cm^{-1} upon protonation and shifts only to 1614 cm^{-1} in the deuterated form (Foglizzo & Novak, 1970). Thus the Raman enhancement pattern strongly supports the assignment of the oxidized flavin mode at 1584 cm^{-1} to a breathing mode of ring II, shifting to 1616 cm^{-1} in the neutral semiquinone.

The long-wavelength CARS spectrum of flavodoxin semiquinone, Figure 3, also shows the 1616-cm^{-1} band, shifted to 1611 cm^{-1}. This shift is similar to that observed for the 1584-cm^{-1} oxidized FMN band, shifting to 1577 cm^{-1} in flavodoxin. The prominent semiquinone band at 1232 cm^{-1} may also be assignable to ring II. It disappears when the semiquinone is prepared in D_2O, resulting in the replacement of the N_5 proton by a deuteron, suggesting a substantial contribution from N_5–H bending. Pyrazinium ion has a similar mode at 1214 cm^{-1} which shifts to 918 cm^{-1} upon deuteration (Foglizzo & Novak, 1970). (The 900-cm^{-1} region was inaccessible in our semiquinone CARS spectrum because the small crossing angle required for phase matching when ω_1 is at long wavelengths produces excessive stray light when $\Delta\omega < 1000$ cm^{-1}.) The prominent pair of bands at 1391 and 1378 cm^{-1} may be correlated with the ring II and III modes (Dutta et al., 1980) at 1409 and 1356 cm^{-1} in oxidized flavodoxin. They are replaced by a single band at 1386 cm^{-1} in D_2O. The semiquinone has a cluster of weak bands between 1300 and 1340 cm^{-1}, resembling that of oxidized flavodoxin between 1220 and 1290 cm^{-1}, although one of the latter group may correlate with the semiquinone band at 1269 cm^{-1}. Unfortunately, the D_2O sensitivity of the weaker semiquinone bands cannot be ascertained because the CARS spectrum in D_2O had an appreciably lower signal-to-noise ratio.

Conclusions

(1) Oxidized flavodozin gives an excellent quality resonance CARS spectrum. Modest frequency lowerings, relative to aqueous FMN, of three prominent bands are attributable to environmental effects at the protein binding site. The strength of the N_3H H bond to Glu-5 is comparable to that of FMN to water.

(2) The CARS spectrum of flavodoxin semiquinone is very different from that of the oxidized form and serves as a useful fingerprint. It contains prominent bands at 1611 and 1232 cm^{-1} assignable to the protonated ring II, by analogy with the pyrazinium ion.

(3) Both oxidized flavin and the neutral semiquinone show only one enhanced CARS band, that of the pyrazine breathing mode at 1584 and 1616 cm^{-1}, respectively, when excited in their shorter wavelength absorption bands, in contrast to the

multiple enhancements observed for the longer wavelength bands. This parallelism supports the assignment of the absorption bands to the same pair of π–π^* transitions, shifted to lower energy for the semiquinone.

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Solubilization and Characterization of Apolipoprotein B from Human Serum Low-Density Lipoprotein in *n*-Dodecyl Octaethylene Glycol Monoether†

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ABSTRACT: Apolipoprotein B, the core polypeptide of human serum low-density lipoprotein, retains its native association state (500 000 g/complex), as well as its native conformation as judged by circular dichroism, when all lipid has been replaced by a nonionic detergent. Protein solubilized in this detergent should therefore be well suited for lipid binding studies. The native association state is also preserved when lipid is replaced by ionic detergents, but in this case the protein undergoes a conformational change, which can be reversed if the ionic detergent is replaced by nonionic detergent. The constancy of the state of association of the B polypeptide in a variety of amphiphilic environments contrasts with what has been observed with polypeptides from high-density lipoproteins which exist in different states of association under different conditions.

Low density lipoproteins (LDL),[1] the main cholesteryl ester transport vehicles in human serum, arise in the plasma as catabolic products of the triglyceride-rich very low density lipoproteins (VLDL). This metabolic processing gives rise to two distinct subclasses of LDL termed LDL_1 and LDL_2 with density ranges of 1.006–1.019 g/cm^3 and 1.02–1.063 g/cm^3, respectively. LDL_2 is composed of 78% lipid and 22% protein, the latter consisting exclusively of a single polypeptide, apolipoprotein B (apo-B). In all classes of LDL and VLDL that

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[1] Abbreviations used: LDL, human low-density serum lipoprotein; VLDL, human very low density serum lipoprotein; $NaDodSO_4$, sodium dodecyl sulfate; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether.

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