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THE EH_2 REDUCED INTERMEDIATE OF GLUTATHIONE REDUCTASE CONTAINS OXIDISED FLAVIN-WHILE EH_4 DOES NOT

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Glutathione reductase is a flavoprotein whose x-ray structure has been established. Functional data and the x-ray structure are consistent with a mechanism of reaction in which NADPH reacts with the enzyme to produce a two electron, EH_2, and four electron, EH_4, intermediate. The former is competent for the transfer of electrons to the substrate glutathione. Several structures are possible for the two NADPH intermediates; in order to aid in the determination of the structure of these intermediates, we have determined their resonance Raman spectra at two excitation frequencies. These studies establish that the EH_2 intermediate is an oxidized flavin species while the EH_4 species is not. Furthermore, the most likely structure for EH_2 involves a charge transfer donation of electrons from the anion of cys-63 to the N_5 position of flavin.

INTRODUCTION: Glutathione Reductase is a flavoprotein which catalyzes the reduction of glutathione by NADPH.[1] The x-ray structure of the enzyme has been determined, and the structural data suggest a mechanism in which electrons move through the flavin to a cystine disulfide in the active site and ultimately to the substrate- glutathione.[2] In the process of electron transfer in the absence of glutathione, two intermediates are formed upon addition of the reducing agent, NADPH, to the enzyme. The first of these is the so called EH_2 intermediate; this species exhibits an electronic spectrum with some (approximately 40%) bleaching in the 450 nm $\pi-\pi^*$ electronic band along with production of a long wavelength transition at approximately 570 nm[3]. The latter may be a charge transfer band. There has been much discussion of the structure of the EH_2 intermediate since it is kinetically competent for reaction with glutathione. Possible structures are a biradical of flavin semiquinone and sulfur radical, a covalent adduct of the thiolate anion to the flavin and a charge transfer complex with thiolate anion donor

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and oxidized flavin acceptor. The biradical is unlikely because the electronic spectrum of the two commomn flavin radicals is quite different from the spectrum of EH_2 [4]; similarly, the adduct would be expected to exhibit a very different spectrum from that observed [5]. Further reduction of EH_2 produces an EH_4 species which exhibits approximately 80% bleaching of the 450 nm electronic band of flavin; this species most likely contains a reduced flavin hydroquinone.

Resonance Raman, RR, spectroscopy has proven a valuable method for the investigation of flavin structure; isotopic substitution [6], normal coordinate analysis [7], and model studies [8] have led to an understanding of the spectrum of oxidized flavin and the effect of bonding interactions upon the RR spectrum of oxidized flavoproteins [8]. In addition the RR spectrum of the blue neutral semiquinone form of flavin[9, 10], and the spectrum of the reduced flavin in the hydroquinone form of the charge transfer complex of D-amino acid oxidase have been determined [11]. It is clear from these latter studies that the RR spectrum of flavoproteins offers a very good method to determine the oxidation state of the flavin in enzyme intermediates. Accordingly, we have determined the RR spectrum of the the reduced intermediates of Glutathione Reductase; we have previously reported the spectrum of the oxidized enzyme.

MATERIALS AND METHODS: Glutathione reductase was purchased from Sigma Chemical Co. as a purified suspension in an ammonium sulfate solution. This suspension was dialyzed for 24 hr. in a 50 mM phosphate (pH 8.0) buffer containing 250 mM sulfate as an internal standard for the Raman spectroscopy. The enzyme was then concentrated using a Schleicher & Schuell collodion bag concentrator; the concentration was measured spectrally using the 450 nm flavin absorbance.

The two electron reduced enzyme, EH_2, was prepared by adding a ten fold excess of NADPH to the glutathione reductase; this addition was carried out in a glove bag with an atmosphere of N_2 to insure anaerobicity. The excess NADPH was added in order to remove any residual O_2 remaining in the buffer. Approximately 25 μl of the EH_2 solution was placed in a capillary tube; which was sealed in the glove bag to avoid oxygen contamination. The resonance Raman spectra were obtained as described previously[8].

The four electron reduced enzyme, EH_4, was prepared by addition of excess sodium arsenite and NADPH to the glutathione reductase solution. The arsenite forms a complex with the active site cysteines of glutathione reductase thus permitting complete reduction of the FAD by NADPH. The reaction was performed in an N_2 glove bag and the enzyme solution was placed in a capillary tube as described above for EH_2.

RESULTS AND DISCUSSION: Figure I shows the RR spectra of oxidized glutathione reductase, E, and of EH_2 at two wavelengths. Table I compares the frequencies of the various bands in the EH_2 and oxidized forms of the enzyme. It is clear from the spectra and the data in Table I that the EH_2 intermediate is an oxidized flavin species. Bands I-XIII are clearly visible in the same frequency region in EH_2 and oxidized enzyme. We find no band, which can be identified with the charge transfer donor; all of the observed vibrational bands appear to be from flavin. We do not observe a νC-S band in the 600-700 cm^{-1} region of the spectrum.[16] Furthermore, the comparison of the flavin RR spectra in oxidized enzyme and the EH_2 intermediate is consistent with comparable hydrogen bonding between the flavin and protein in EH_2 and in oxidized enzyme. This determination is based on the similarity of the positions of Bands II and X in these two species. These RR bands are sensitive to hydrogen bonding changes at the N_1, N_5 and N_3-H, C_2=O, C_4=O respectively[8]. There is one major difference between the RR spectrum of the oxidized enzyme and EH_2-the presence of an additional band in the oxidized enzyme. This band is found between Band X and XI and disappears upon reduction; a similar band with the same frequency has been observed in Glucose Oxidase and a flavoprotein isolated from P. leiognathi[12]. All of the flavoproteins showing this extra band are characterized by a resolved electronic transition on the red side of the 450 nm π-π^* transition. The extra band may result from the coupling with this electronic transition; reduction of glutathione reductase reduces the intensity of this electronic transition and may, for this reason, result in the loss of the extra vibrational band. In figure I, flavin has been reduced with a small excess of NADPH to yield the NADP complex of EH_2. There is a large change in intensity of Bands II and III as the excitation frequency is shifted toward the charge transfer band of the NADP complex. Figure II shows the intensities of the various RR peaks for oxidized enzyme and for the EH_2 NADP complex. Comparison of the spectra for the oxidized enzyme with those of the EH_2-NADP complex indicates that bands II and III have undergone significant resonance

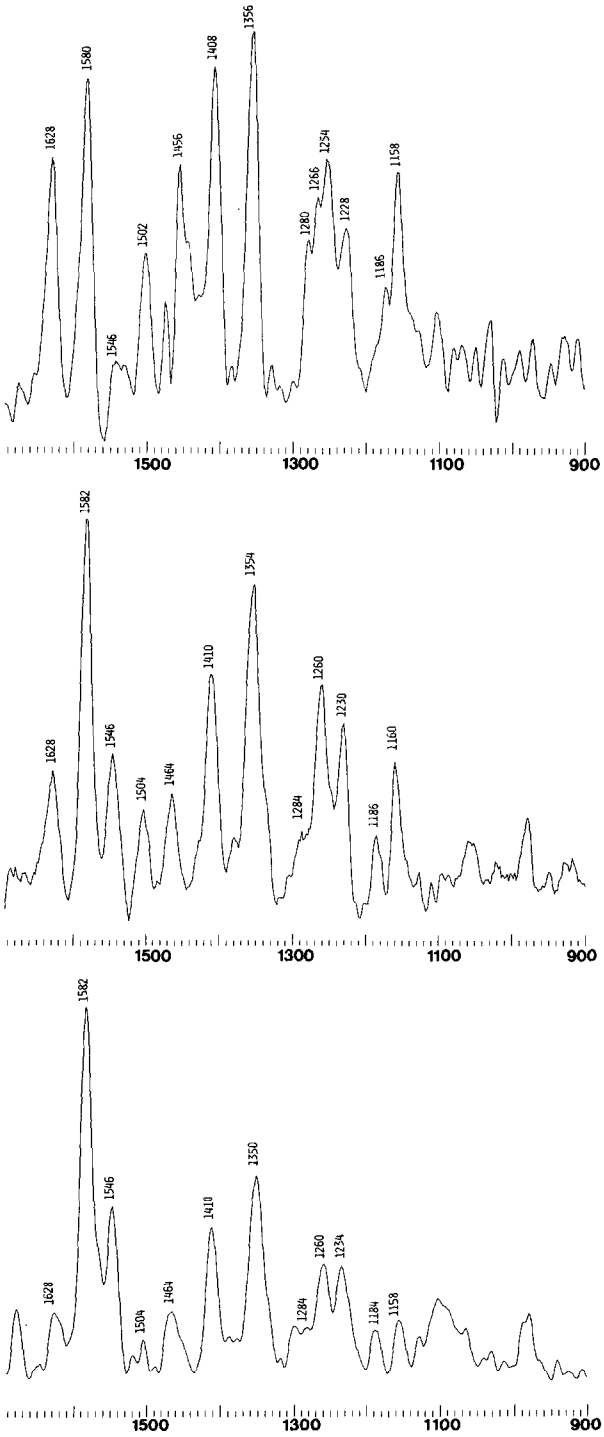

Figure I: Resonance Raman spectra of gluthione reductase and two EH_2 intermeadiates.The spectra are: top) 300 μM oxidized glutathione reductase with laser exciting line of 515 nm, middle) 300 μM glutathione reductase reduced with 10x NADPH, the exciting line was 488 nm, bottom) same solution as in the middle spectrum except the exciting line was 515 nm. The spectra were recorded at 2 cm^{-1} intervals and all solutions contained 250 mM sodium sulfate.

Table I: Comparison of the resonance Raman frequencies of the various bands in the different forms of gluathione reductase

Band	E	EH_2	
		reduced with NADPH	
	λ_o=515nm	λ_o=488nm	λ_o=515nm
I	1628(4.00)	1628(1.95)	1628(0.94)
II	1580(5.28)	1582(5.60)	1582(6.18)
III	1546(0.48)	1546(2.25)	1546(2.88)
IV	1502(2.32)	1504(1.40)	1504(0.41)
V	1456(3.76)	1464(1.55)	1464(1.71)
VI	1408(5.48)	1410(3.30)	1410(2.59)
VII	1356(6.08)	1354(4.50)	1350(3.59)
IX	1280(2.60)	1284(0.31)	1284(1.11)
X	1266(3.32)	1260(2.70)	1260(1.18)
1254	1254(4.00)		
XI	1228(2.80)	1230(2.35)	1234(2.00)
XII	1186(1.76)	1186(1.25)	1184(0.82)
XIII	1158(3.76)	1160(2.35)	1158(0.94)

All frequencies are in units of cm^{-1} and are relative to a sulfate standard of 981 cm^{-1}. λ_o line is the laser exciting line wavelength. The numbers in parenthesis (rel I) are the intensities relative to a 250 mM sulfate standard in each of the solutions.

enhancement upon irridation at 515 nm. Most of the RR bands (I, IV, VI, VII, X, XI, XII, XIII) of the EH_2-NADP complex show the expected decrease in intensity as the excitation frequency is moved away from the 450 nm π-π^*

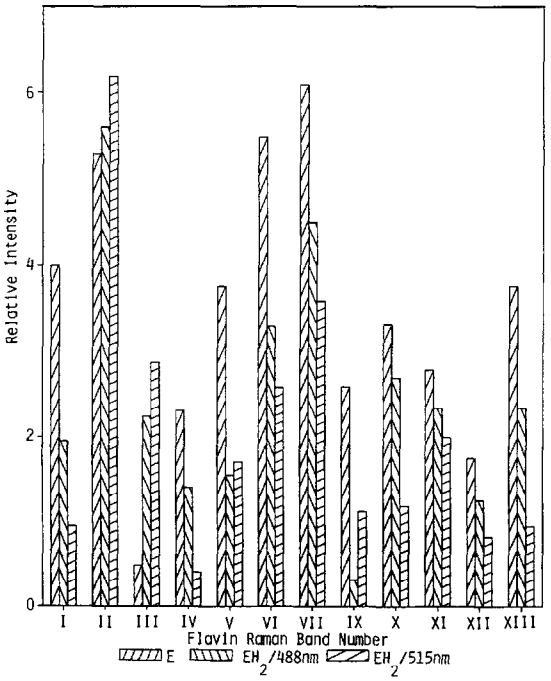

Figure II: The relative intensities of the various RR bands of the different glutathione reductase species. The intensities are relative to the intensity of the 981 cm^{-1} sulfate band.
E 515nm: figure I top spectrum
EH2 488nm: figure I middle spectrum
EH2 515nm: figure I bottom spectrum

transition. However, bands II, III, and IX increase in intensity upon long wavelength excitation, Figure II. These data indicate that the coupling of bands II, III and IX of EH_2 to the long wavelength transition can be interpreted in terms of the structure of the charge transfer complex in EH_2. Charge transfer complexation is usually observed as an electronic excited state event in which there is little redistribution of charge in the electronic ground state [13]. Our observations on the charge transfer complexes of Fatty Acyl-CoA Dehydrogenase are consistent with this interpretation since there is no shift in RR spectral band position indicating no bond reorganization in the ground state, but there is significant change in band intensity[14]. The latter would be expected in an excited state charge transfer complex since the equation for RR intensity contains a term in the denominator which includes the difference in frequency between the electronic transition under investigation and the excitation frequency of the laser. As a first approximation, the bands showing maximum change in RR intensity are those associated with parts of the molecule that undergo the largest degree of bond reorganization associated with the charge transfer. As the excitation frequency is moved toward the charge transfer electronic transition for the EH_2-NADP complex, Bands II, III and IX show the large increases in intensity. Since Band II and III are associated with $\nu C_{4a}{=}N_5$ and $\nu C_{10a}{=}N_{10}$ and since the N_5 position is the most electronegative[15], the most likely structure for the charge transfer complex involves electron donation from the thiolate anion of cysteine to the N_5 position of oxidized flavin. The other band showing resonance enhancement, band IX, exhibits a shift in vibrational frequency in D_2O indicating that it is associated with N_3-H. This band is also in the region of a vibrational mode for uricil which involves the carbonyl group at position 2 and 4. Therefore, a possible assignment for this band is an out of plane bending mode for $C_2{=}O$, $C_4{=}O$ coupled to the N_3-H bending mode. These considerations of vibrational assignments lead to the conclusion that charge transfer occurs in the N_5, C_{4a}, $C_4{=}O$ region of the flavin molecule and might involve aromatic

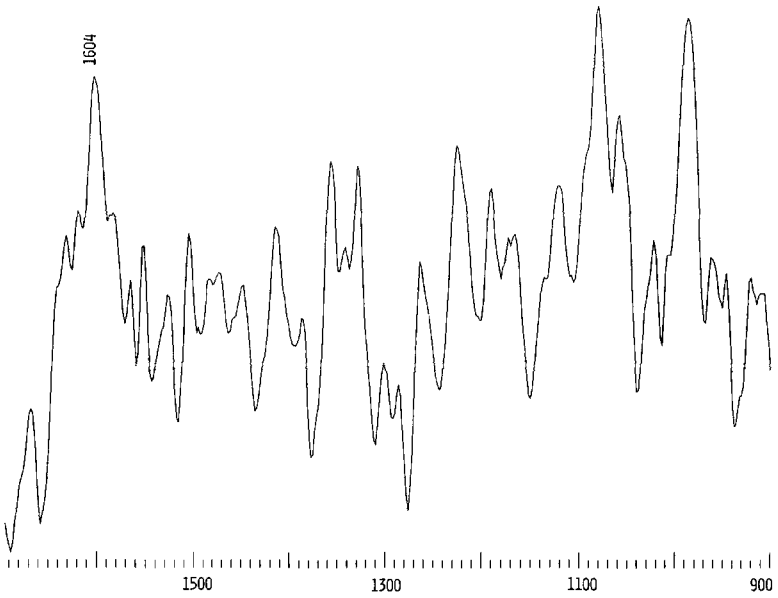

Figure III: Resonance Raman spectrum of the four electron reduced glutathione reductase, EH4. The solution contained 300 μM glutathione reductase, 10x excess $NaAsO_2$, and 10x excess NADPH. The spectrum was recorded at 2 cm^{-1} intervals with laser excitation at 515 nm.

delocalization of the donated electron to the N_5, C_{4a}, C_4 positions of the flavin molecule. These observations and interpretations on the charge transfer complexes of Glutathione Reductase are simular to those on the acetoacetyl-CoA complex of Fatty Acyl-CoA Dehydrogenase[14].

Figure III shows the RR spectrum of the EH_4 species of Glutathione Reductase; although the signal to noise ratio is not what we would like, we can identify two vibrational bands (1604 cm^{-1} and 1350 cm^{-1}) which we have seen in all of these spectra. The first of these is located at the same frequency as a band in the RR spectrum of a charge transfer complex of D-Amino Acid Oxidase which has been identified as a charge transfer complex of imino acid with the reduced flavin hydroquinone[11]. Our preliminary conclusion is that EH_4 contains reduced flavin hydroquinone. We currently are trying to obtain better RR spectra of the intermediate by employing a longer wavelength excitation frequency.

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