The resonance Raman spectra of the oxidized and two-electron reduced forms of yeast glutathione reductase are reported. The spectra of the oxidized enzyme indicate a low electron density for the isoalloxazine ring. As far as the two-electron reduced species are concerned, the spectral comparison of the NADPH-reduced enzyme with the glutathione- or dithiothreitol-reduced enzyme shows significant frequency differences for the flavin bands II, III, and VII. The shift of band VII was correlated with a change in steric or electronic interaction of the hydroxyl group of a conserved Tyr with the N$_{10}$-$C_{10a}$ portion of the isoalloxazine ring. Upward shifts of bands II and III observed for the glutathione- or dithiothreitol-reduced enzyme indicate both a slight change in isoalloxazine conformation and a hydrogen bond strengthening at the N$_5$ and/or N$_7$ site(s). The formation of a mixed disulfide intermediate tends to slightly decrease the frequency of bands II, III, X, XI, and XIV. To account for the different spectral features observed for the NADPH- and glutathione-reduced species, several possibilities have been examined. In particular, we propose a hydrogen bonding modulation at the N$_5$ site of FAD through a variable conformation of an ammonium group of a conserved Lys residue. Changes in N$_5$(flavin)-protein interaction in the two-electron reduced forms of glutathione reductase are discussed in relation to a plausible mechanism of the regulation of the enzyme activity via a variable redox potential of FAD.

Glutathione reductase (GR,$^1$ EC 1.6.4.2, NADPH:oxidized glutathione oxidoreductase) is a pyridine nucleotide-disulfide oxidoreductase that shares many spectroscopic and structural properties with other members of this family such as lipoamide dehydrogenase, thioredoxin reductase, NADH peroxidase, and mercuric reductase (1). GR is a cytoplasmic flavoenzyme that is widely but not universally distributed in aerobic organisms. As far as the two-electron reduced species are concerned, the spectral comparison of the NADPH-reduced enzyme with the glutathione- or dithiothreitol-reduced enzyme shows significant frequency differences for the flavin bands II, III, and VII. The shift of band VII was correlated with a change in steric or electronic interaction of the hydroxyl group of a conserved Tyr with the N$_{10}$-$C_{10a}$ portion of the isoalloxazine ring. Upward shifts of bands II and III observed for the glutathione- or dithiothreitol-reduced enzyme indicate both a slight change in isoalloxazine conformation and a hydrogen bond strengthening at the N$_5$ and/or N$_7$ site(s). The formation of a mixed disulfide intermediate tends to slightly decrease the frequency of bands II, III, X, XI, and XIV. To account for the different spectral features observed for the NADPH- and glutathione-reduced species, several possibilities have been examined. In particular, we propose a hydrogen bonding modulation at the N$_5$ site of FAD through a variable conformation of an ammonium group of a conserved Lys residue. Changes in N$_5$(flavin)-protein interaction in the two-electron reduced forms of glutathione reductase are discussed in relation to a plausible mechanism of the regulation of the enzyme activity via a variable redox potential of FAD.

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\[
\text{GSSG} + \text{NADPH} + H^+ \rightleftharpoons 2 \text{GSH} + \text{NADP}^+ \quad (\text{Eq. 1})
\]

By maintaining an elevated [GSH]/[GSSG] ratio in most eu-
In this reaction, many intermediates are formed. A stable mixed disulfide (MDS) intermediate was proposed according to the following equations (7).

\[
\text{EH}_2 + \text{GSSG} \rightleftharpoons \text{EH}_2\text{-GSSG} \rightleftharpoons \text{MDS-GSH} \rightleftharpoons \text{MDS} + \text{GSH} \quad (\text{Eq. 4})
\]

\[
\text{MDS} \rightleftharpoons \text{E}_{ox}\text{-GSH} \rightleftharpoons \text{E}_{ox} + \text{GSH} \quad (\text{Eq. 5})
\]

In this catalytic scheme, the Cys-58 residue (human GR) is the interchange (or distal) thiol and interacts with GSSG (1, 7). The acid-base catalyst of the interchange, i.e. the histidyl side chain of His-467 paired with a glutamate residue (Glu-472), would have two roles; its primary function would be to protonate (acid catalyst) the first departing molecule of GSH, inhibiting the back reaction, i.e. attack of glutathione thiolate on the MDS (10). The second function (base catalyst) would be to stabilize the charge-transfer complex between FAD and the thiolate group of Cys-63 (6). Therefore, the interchange thiol, the charge-transfer thiol, and the His-Glu acid-base catalyst are closely linked structural elements in human GR. They are conserved in the E. coli and yeast sequences (1, 11, 12).

A full understanding of the molecular mechanism of electron transfer in GR requires detailed information concerning the structures and environments of the redox sites. To better understand the role of the FAD cofactor in GR, we have undertaken a resonance Raman (RR) study on the two-electron reduced state, this redox state appearing to be of key importance in the function and regulation of the enzyme (4, 6, 7). RR spectroscopy has proven to be a valuable method for the investigation of the structure and the environment of the flavins in flavoproteins (13, 14).

In this paper, we describe and analyze the RR spectra of the \( \text{E}_{ox}\text{-EH}_2 \) and MDS forms of the yeast GR. The two-electron reduced states were formed under controlled conditions using different reducing agents: NADPH, GSH, or dithiothreitol (DTT), in the absence or presence of GSSG or NADP\(^+\). Although the three-dimensional structure of oxidized GR is known, and models of the reductive and oxidative half-reactions are available, many questions still remain unanswered. One of these is the importance of FAD in the GR catalysis. Our objective is to determine the possible influences of the substrates and products on the structure and environment of the isoalloxazine ring of FAD and to relate our observations to the function and regulation of the GR activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Yeast glutathione reductase was purchased from Sigma. The enzyme in an ammonium sulfate solution was dialyzed for 6 h in 50 mM potassium phosphate buffer, 0.5 mM EDTA, pH 7.2. To improve the protein purity, the solution was then applied onto a column of 2 mL Sepharose (Amersham Biosciences) (15). The elution of GR was done with a linear NaCl gradient (0–1 M) added to the buffer (8). The pooled fractions were washed by repeated concentrations and dilutions with 10 mM potassium phosphate buffer, 0.5 mM EDTA, pH 7.2, on Centricon 50. The concentration of the eluted oxidized enzyme was measured and the activity of the purified enzyme was calculated using \( \text{E}_{ac} = 6.220 \times 10^{-1} \text{ cm}^{-1} \) at 462 nm (16). The measured activities of the purified enzyme in an ammonium sulfate solution were higher than 0.33 mmol of NADPH/min/mg of protein. The NADPH concentration was calculated using the equation \( \text{NADPH} = \frac{\text{NADPH}}{2} \times \text{GSH} \times \frac{1}{100} + \frac{\text{GSH}}{2} \times \frac{1}{100} \) (17).

The two-electron reduced forms of the enzyme, EH\(_2\), and MDS were generated by reduction of oxidized GR (0.6–0.8 mM for the Raman experiments) with NADPH, GSH, or DTT in the presence or absence of GSSG or NADP\(^+\). These reductions were performed under anaerobic conditions in equilibrium with wet argon (18). After equilibration, a small aliquot of a solution of NADPH or GSH was injected through a septum in a cuvette containing the solution of GR. The color change of the enzyme from yellow to orange-red as well as the concomitant changes in absorption spectrum proved the formation of the EH\(_2\) or MDS species. To maintain the enzyme in the two-electron reduced state, the spectroscopic measurements were performed under anaerobic conditions using a continuous flow of wet argon over the surface of the sample. Under these conditions, the EH\(_2\) and MDS samples were stable for several hours. The reduction of GR with DTT was conducted by dialyzing the protein at 4 °C in a 50 mM potassium phosphate buffer, pH 7.2, containing 0.5 mM DTT. This reduction produces an EH\(_2\) species that is stable for several hours, even in the presence of air.

Using electronic absorption and RR spectroscopies, the enzyme stability under laser irradiation was checked. No spectral variation was observed during the experiments. After the RR experiments were done, we controlled the reversibility of the enzyme reduction in regenerating the fully oxidized form by dialysis and in measuring the enzyme activity. Such controls showed that the GR activity was stable during the spectral determinations.

**Spectroscopy**—Resonance Raman experiments were recorded at 20 ± 1 °C on a Jobin-Yvon spectrometer (Ramanor HG2S-UV) using argon (Coherent, model Innova 100) and helium/cadmium (Licoix, model 4050) lasers. The signal-to-noise ratios were improved by summation of several hours, even in the presence of air.

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**RESULTS**

**RR Spectra of Oxidized GR**—Fig. 1a shows the high frequency region of the RR spectrum of oxidized GR, excited at 441.6 nm. In this study, the band numbering previously adopted for the prominent RR bands of oxidized flavoenzymes has been used (20). In the 1300–1700 cm\(^{-1}\) region of the spectra, four bands observed at 1626 (band I), 1579 (band II), 1402 (band VI), and 1353 (band VII) cm\(^{-1}\) dominate. However, shoulders on the high frequency sides of band I (at 1643 cm\(^{-1}\) and 1654 cm\(^{-1}\)) and band IV (at 1489 cm\(^{-1}\)) are also identified. The 1200–1300 cm\(^{-1}\) region of the RR spectrum shows a complex pattern with overlapping bands at 1224, 1248, 1264, and 1275 cm\(^{-1}\) (Fig. 2a). The frequency of the RR bands observed in the 1000–1700 cm\(^{-1}\) region of oxidized GR (\( \text{E}_{ox} \)) are listed in Table I. The addition of either GSSG (up to 60 eq) or NADP\(^+\) (up to 100 eq) to oxidized GR has no influence on the RR spectra (spectra not shown).

**RR Spectra of NADPH-reduced GR**—The RR spectra of GR anaerobically reduced with NADPH (2.5 eq) at pH 7.2 are

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\(^2\) EH\(_2\) is generally considered as a mixture of several two-electron reduced species, including MDS. EH\(_2\) will be used here to mean all two-electron reduced species other than MDS.
Raman Studies of Yeast Glutathione Reductase

Fig. 2. 1100–1300 cm⁻¹ regions of RR spectra of GR at pH 7.2. a, oxidized GR. b, GR reduced with 2.5 eq of NADPH. c, GR reduced with 20 eq of GSH. d, GR reduced with 2 eq of DTT. Excitation was 441.6 nm. Summations were of 10–30 scans. a.u., —.

displayed in Figs. 1b and 2b. When compared with the RR spectra of the oxidized form (Figs. 1a and 2a), those of the NADPH-reduced GR have lower signal-to-noise ratios. However, we clearly identify significant shifts for bands II (+2 cm⁻¹), V (+3 cm⁻¹), VI (+5 cm⁻¹), and VII (−2 cm⁻¹). The 1200–1300 cm⁻¹ regions also exhibit substantial modifications. The band observed at 1248 cm⁻¹ for oxidized GR is markedly diminished upon enzyme reduction, whereas we notice an increase in relative intensity and a broadening of the 1262 cm⁻¹ band for the NADPH-reduced form (Fig. 2b). Band XI is apparently upshifted from 1224 cm⁻¹ for oxidized GR to 1230 cm⁻¹ for NADPH-reduced GR (Fig. 2, a and b).

When GR is reduced by NADPH (2 eq) at pH 7.2, the addition of NADP⁺ (15–150 eq) has no significant influence on the frequencies of the RR bands (Fig. 1c and Table I). However, a slight broadening of these bands is detected.

**RR Spectra of GSH- and DTT-reduced GR**—The titration of GR with GSH produces gradual upshifts of the RR bands II, III, V, VI, IX, and X (spectra not shown). With a saturating concentration of GSH (15–20 eq), the RR spectrum of the GSH-reduced enzyme exhibits maximal shifts of +3–6 cm⁻¹ for bands II, III, V, and VI, when compared with the spectrum of oxidized GR (Fig. 3a versus Fig. 1c and Table I). In the case of the NADPH-reduced enzyme, band X is apparently upshifted from 1248 to 1262 cm⁻¹ (Fig. 2, a and c). The comparison of the 1300–1670 cm⁻¹ regions of the spectra of NADPH-reduced GR and GSSG-reduced GR shows frequency shifts for bands II (from 1581 to 1584 cm⁻¹), III (from 1546 to 1549 cm⁻¹), and VII (from 1351 to 1354 cm⁻¹) (Fig. 1b versus Fig. 3a). In the 1200–1300 cm⁻¹ regions, the RR spectrum of the GSH-reduced enzyme closely resembles that of the NADPH-reduced form (Fig. 2b and c and Table I). The RR spectrum of GR reduced with DTT at pH 7.2 is essentially identical to that of the enzyme reduced with GSH (Figs. 2a and 3b). The broad band XIV, however, appears to be downshifted (Table I).

Combined effects of GSH, GSSG, and pH produce different ratios of EH₂ and MDS (7). The RR spectrum of GR reduced with GSH at pH 8.4 is shown in Fig. 3c. The RR spectra of GR reduced with GSH (15 eq) at pH 6.3 in the presence of various concentrations of GSSG (2–60 eq) are not significantly different from each other (spectra not shown). The observed frequencies are listed in Table I, and one of these spectra is displayed in Fig. 3d. The comparison of the RR spectrum of GR reduced with GSH at pH 8.4 with that of GR reduced with GSH at pH 6.3 in the presence of GSSG shows a significant downshift and broadening of bands II (from 1585 to 1583 cm⁻¹) and III (from 1551 to 1548 cm⁻¹) (Fig. 3, c and d). In the lower frequency regions, bands X (1263/1261 cm⁻¹), XI (1229/1227 cm⁻¹), and XIV (1061/1058 cm⁻¹) are also slightly affected (spectra not shown and Table I).

We have investigated the effect of NADP⁺ on the GSH-reduced enzyme at pH 7.2. The RR spectrum obtained for GR reduced with GSH (15 eq) at pH 7.2 in the presence of NADP⁺ (15 eq) is very similar to the spectrum obtained for GR at the same pH in the absence of NADP⁺. Small differences concern a slight broadening of most of the RR bands and the detection of a downshift of band II from 1584 cm⁻¹ in the absence of NADP⁺ to 1583 cm⁻¹ in its presence (spectra not shown and Table I).

**DISCUSSION**

**RR Spectra of the Eox,EH₂ and MDS Species of Yeast GR**

**Oxidized GR**—The assignment of the RR bands of the isoalloxazine ring is based on isotopic data and normal mode vibrational analysis (19–23). Fig. 4 shows the chemical structure and the numbering of the isoalloxazine ring, and Table I summarizes the band assignments for the oxidized and two-electron reduced forms of yeast GR. The RR spectra of oxidized GR were previously investigated (24, 25). Our study shows frequencies close to those published in the study of Schmidt et al. (24). With reference to the RR frequencies of FAD in water, Raman diagrams were previously drawn to visualize the apoprotein effect on the high frequency modes of the isoalloxazine ring (19, 26). In the diagram of oxidized GR (Fig. 5), the frequency shifts detected for GR are generally negative, indicating an electron-deficient isoalloxazine ring (19, 26). The shapes of the Raman diagrams were associated with the electron distribution through the three rings of the isoalloxazine moiety and correlated with the biological activities of several flavoproteins (19, 26). The profile of GR is very different from those previously obtained for electron transferases or oxidases (19, 26).

**NADPH-reduced GR**—The reduction of GR with NADPH provides an EH₂ complex (Equation 2). The reaction product, NADP⁺, forms a complex with EH₂ ($K_D = 70 \mu M$), but NADPH in excess forms a tighter EH₂–NADPH complex ($K_D = 21 \mu M$) (27). This latter complex enhances the thiolate-flavin CT band (9).

The RR spectra of NADPH-reduced GR are in agreement with an oxidized isoalloxazine ring (Table I) (13, 14, 19, 25). The comparison of the RR diagrams drawn for oxidized GR and its NADPH-reduced form illustrates the influence of the disulfide reduction on the isoalloxazine modes (Fig. 5). Upshifted frequencies are observed for most of the RR bands of the NADPH-reduced GR, indicating an increased electron density of the isoalloxazine ring upon dithiol formation. However, the isoalloxazine ring remains electron-deficient. This low electron density is likely at the origin of the stable formation of the CT complex between FAD and the proximal thiolate group constituting the dithiol active site (1). The changes in shape of the Raman diagrams also indicate a modification in the electron distribution through the isoalloxazine ring (Fig. 5).

For an enzyme preliminarily reduced with excess NADPH (EH₂–NADPH), the displacement of the bound NADPH molecule by NADP⁺ has no influence on the RR modes of FAD (Table I). Although NADPH binding causes an important protein reorganization around Tyr-197 (2, 4), the structure and environment of the FAD do not therefore discriminate the
presence of either NADPH or NADP+ in the NADPH-binding site of EH2.

GSH- and DTT-reduced GR—Glutathione is the physiological product of the GR catalysis. With a redox potential (E°) of −234 mV, it is capable to reduce oxidized GR (1, 7, 16, 28). This reaction does not represent the reversal of Equation 3. It was suggested that the back reaction is blocked at the MDS stages (Equations 5 and 4) (7, 28). Direct evidence for formation of MDS was provided by x-ray crystallography using human GR crystals soaked with GSH (2, 4). The MDS-GSH species accumulates when GSSG is added and/or pH is decreased (7). The absorption spectrum of MDS appears to maintain an absorbance at 540 nm very similar to that of the CT complex (7, 28).

DTT can reduce GR, considering the absorption spectrum of DTT-reduced GR that exhibits the 540 nm CT band (spectrum not shown). The formation of an EH2 state by opening of the disulfide redox center of GR (E° = −242 mV) is expected considering the redox potential of DTT (E° = −327 mV) (1, 8, 29).

### Table I

| RR band | E° | NADPH, pH 7.2 | DTT, pH 7.2 | GSH, pH 8.4 | GSH, pH 7.2 | GSH/GSSG, pH 6.3 |
|---------|----|---------------|-------------|-------------|-------------|------------------|
| I       | 1643 | 1645          | 1628        | 1627        | 1627        | 1643             |
| II      | 1579 | 1581          | 1584        | 1584        | 1583        | 1583             |
| III     | 1545 | 1546          | 1551        | 1549        | 1548        | 1548             |
| IV      | 1498 | 1500          | 1501        | 1499        | 1500        | 1464             |
| V       | 1461 | 1464          | 1454        | 1464        | 1463        | 1463             |
| VI      | 1402 | 1408          | 1354        | 1353        | 1355        | 1355             |
| VII     | 1353 | 1351          | 1354        | 1279        | 1276        | 1278             |
| VIII    | 1273 | 1251          | 1329        | 1263        | 1262        | 1261             |
| IX      | 1248 | 1248          | 1263        | 1279        | 1276        | 1278             |
| X       | 1224 | 1227          | 1227        | 1229        | 1226        | 1227             |
| XI      | 1181 | 1183          | 1182        | 1182        | 1183        | 1182             |
| XII     | 1156 | 1156          | 1154        | 1158        | 1156        | 1157             |
| XIII    | 1124 | 1115          | 1153        | 1133        | 1132        | 1132             |
| XIV     | 1058 | 1058          | 1055        | 1061        | 1059        | 1058             |

**Assignment**

Assignment in Table I is based on Table I of Refs. 20–22.

![Fig. 3](#) 1300–1670 cm⁻¹ regions of RR spectra of GR reduced by GSH or DTT. a, GR reduced with 20 eq of GSH at pH 7.2. b, GR reduced with 2 eq of DTT at pH 7.2. c, GR reduced with 20 eq of GSH at pH 8.4. d, GR reduced with 20 eq GSH at pH 6.3, in the presence of 60 eq GSSG. Excitation was 441.6 nm. Summations were of 15–40 scans. The *asterisks* above the spectra indicate plasma lines. a.u., ——.

![Fig. 4](#) Chemical structure and numbering of the oxidized isoalloxazine ring.

The presence of either NADPH or NADP⁺ in the NADPH-binding site of EH2.

**GSH- and DTT-reduced GR**—Glutathione is the physiological product of the GR catalysis. With a redox potential (E°) of −234 mV, it is capable to reduce oxidized GR (1, 7, 16, 28). This reaction does not represent the reversal of Equation 3. It was suggested that the back reaction is blocked at the MDS stages (Equations 5 and 4) (7, 28). Direct evidence for formation of MDS was provided by x-ray crystallography using human GR crystals soaked with GSH (2, 4). The MDS-GSH species accumulates when GSSG is added and/or pH is decreased (7). The absorption spectrum of MDS appears to maintain an absorbance at 540 nm very similar to that of the CT complex (7, 28).

DTT can reduce GR, considering the absorption spectrum of DTT-reduced GR that exhibits the 540 nm CT band (spectrum not shown). The formation of an EH2 state by opening of the disulfide redox center of GR (E° = −242 mV) is expected considering the redox potential of DTT (E° = −327 mV) (1, 8, 29).
The RR frequencies observed for GR reduced at pH 7.2 with either GSH or DTT again correspond to an oxidized flavin ring (Table I) (13, 14, 19). Taking into account the different uncertainties on these frequencies, the diagrams obtained for GR reduced with GSH or DTT are identical but differ from that obtained for NADPH-reduced GR (Fig. 5 and diagram not shown). On one hand, the increases in frequency of bands II and III observed upon NADPH reduction are lower than those detected using either GSH or DTT as a reducer. On the other hand, the frequencies of bands IX–XII are relatively less up-shifted in the spectra of the GSH- or DTT-reduced enzyme (Fig. 5). For the GSH- or DTT-reduced forms, the most important frequency shifts concern bands II (Δ5 cm\(^{-1}\)), III (Δ4–7 cm\(^{-1}\)), VI (Δ5–6 cm\(^{-1}\)), and X (Δ14 cm\(^{-1}\)) (Fig. 5 and Table I). Although the Raman diagrams of the GSH- or DTT-reduced GR show a gain in electron density, it is again worth noticing that the isoalloxazine ring remains electron-deficient in these two-electron reduced states.

**Effects of GSSG and of pH on the GSH-reduced Enzyme—** When GR is reduced by GSH, the dithiol form of EH\(_2\) and the MDS species are in equilibrium (Equations 4 and 5). In GR reduced with GSH at pH 8.4, the imidazole ring of His-467 partially deprotonates, likely limiting the formation of MDS (7). On the contrary, GR reduced with GSH at pH 6.3 in the presence of GSSG can accumulate the MDS species (7). An intermediate situation occurs for GR reduced by GSH at pH 7.2. Considering the RR spectra of GSH-reduced GR at pH 8.4 and 7.2, and in the presence of GSSG at pH 6.3, the expected increase in MDS concentration produces small downshifts of bands II (1585 to 1583 cm\(^{-1}\)), III (1552 to 1548 cm\(^{-1}\)), X (1263 to 1261 cm\(^{-1}\)), and XIV (1061 to 1058 cm\(^{-1}\)) (Table I). These shifts indicate that the structure of the isoalloxazine ring and/or the environments of the N\(_3\)H and either N\(_1\) or N\(_5\) sites are slightly affected by the thiol-disulfide interchange. In the crystallographic structure of human GR, the interchange thiol, the sulfurs of Cys-58, is far from the flavin ring (3, 4). Nevertheless, the binding state of this atom is likely controlled by Cys-63, proximal to the flavin, and His-467. On one hand, the thiolate anion of Cys-63 interacts directly with the flavin ring (2–4). On the other hand, His-467 participates via its imidazole ring to the cleavage of the GSSG substrate and strongly interacts with the N\(_3\)H group of FAD through its carbonyl peptide group (3). Therefore, the isoalloxazine ring of FAD is sensitive to the formation of MDS through slight changes in its electrostatic environment.

**Bands II and III—** The main differences in RR spectra of the EH\(_2\) and MDS species concern the frequencies of bands II and III (Table I). These bands were associated with the N\(_1\) and N\(_5\) frequencies (18). These bands were associated with the N\(_1\) and N\(_5\) frequencies (18). Taking into account the different uncertainties on these frequencies, the diagrams obtained for GR reduced with GSH or DTT are identical but differ from that obtained for NADPH-reduced GR (Fig. 5 and diagram not shown). On one hand, the increases in frequency of bands II and III observed upon NADPH reduction are lower than those detected using either GSH or DTT as a reducer. On the other hand, the frequencies of bands IX–XII are relatively less up-shifted in the spectra of the GSH- or DTT-reduced enzyme (Fig. 5). For the GSH- or DTT-reduced forms, the most important frequency shifts concern bands II (Δ5 cm\(^{-1}\)), III (Δ4–7 cm\(^{-1}\)), VI (Δ5–6 cm\(^{-1}\)), and X (Δ14 cm\(^{-1}\)) (Fig. 5 and Table I). Although the Raman diagrams of the GSH- or DTT-reduced GR show a gain in electron density, it is again worth noticing that the isoalloxazine ring remains electron-deficient in these two-electron reduced states.

**Band VII—** For GR reduced with NADPH, RR band VII is detected at 1351 cm\(^{-1}\). In the spectra of GR reduced with GSH or DTT, the corresponding band is shifted to 1354–1355 cm\(^{-1}\) (Table I). Band VII was assigned to a ring II mode, with a major contribution of N\(_{10}\)-C\(_{10a}\) stretching (20–22) (Fig. 4). The band VII upshift can be attributed to a change in electronic or steric interaction between the hydroxyl group of Tyr-197 (human GR) and the N\(_{10}\)-C\(_{10a}\) portion of the FAD. In the GSH-reduced enzyme, this hydroxyl group is in close contact with the N\(_{10}\)(FAD) atom (3). Upon NADH binding, the aromatic side chain moves away so that the nicotinamide ring can reach the flavin (3). Therefore, the frequencies of bands II, III, and VII appear to distinguish the EH\(_2\) state generated with NADPH from that generated with GSH or DTT.

**Band X—** Band X of oxidized GR was assigned at 1246 cm\(^{-1}\) on the basis of its large sensitivity on N\(_3\) deuteration (19). This band corresponds to a stretching mode of the N\(_3\)-C\(_4\) and C\(_2\)-N\(_3\) bonds and is expected to be sensitive to the hydrogen bonding state of the N\(_3\)H site (19–22) (Fig. 4). In oxidized GR, the N\(_3\)H group of the isoalloxazine ring of one subunit is tightly bound to the carbonyl group of His-467 of the other subunit (3, 5). The apparent upshift of band X from 1248 cm\(^{-1}\) for oxidized GR to 1261–1263 cm\(^{-1}\) for its reduced forms indicates an increased interaction of the N\(_3\)H(FAD) group with the protein and/or the solvent. This effect is likely linked to the 0.25 Å shift of the flavin ring and/or the slight increased bending of the isoalloxazine ring, observed upon E\(_{ox}/EH_2\) transition (4). Our RR data clearly indicate that the N\(_3\)H-protein interaction is stronger in the EH\(_2\) and MDS states than in E\(_{ox}\).

**Structure and Environment of the Isoalloxazine Ring in the EH\(_2\) and MDS States**

The two-electron reduction of GR with either NADPH or GSH leads to EH\(_2\) and/or MDS species, stable intermediates that are extremely important in the catalysis (1, 6, 7). An opening of the disulfide redox center occurs in both the EH\(_2\) and MDS states. The difference between these forms concern the binding state of the interchange thiol that is either free in the EH\(_2\) states or engaged in a disulfide bond with GSH in the MDS states (4).

The RR frequencies observed for NADPH- and GSH-reduced GR indicate the existence of two different EH\(_2\) forms. In particular, the different frequencies of bands II and III indicate that the ring II-ring III junction has a different structure and/or environment (Table I). In this line, the edge formed by the N\(_{10}\), O\(_{6}\), and N\(_3\)H atoms of FAD as well as the si-side of FAD are surrounded by a number of polar or charged groups in interaction with water molecules (3–5). For human GR, the thiol or thiolate groups of the redox center (Cys-58 and Cys-63) and the Lys-66/Glu-201 and His-467 pairs were associated with the catalytic activity of GR (1). All of these residues are conserved in the amino acid sequence of yeast GR (12). To explain the differences in frequency of bands II and III, several possibilities are envisaged as follows.

**FAD-Thiolate Interactions—** A CT complex between the C\(_{4a}\) flavin atom and the (S(thiolate)) donor group of the disulfide/dithiol center has been postulated for the EH\(_2\) and MDS states (4). The RR diagrams show an important deficit in electron density for the isoalloxazine ring of oxidized GR. The positive shift of bands II and III upon NADPH, GSH, or DTT reduction...
is therefore a consequence of a stable electronic interaction between the negative $S$(thiolate) donor and the $C_{4n}-C_{10a}$ junction.

A first hypothesis is to consider that the $S$(thiolate)-FAD interaction differs in the NADPH- and GSH-reduced forms of GR, provoking different electron densities at the $C_{4n}$ and/or $C_{10a}$ atom(s) of FAD. The absorption spectra of the NADPH-, GSH-, and DTT-reduced forms of GR are very similar. In particular, we notice the presence of a CT band at 540 nm (Ref. 16 and this work). An intensification of the CT band was previously detected for the EH$_2$-NADPH complex (9). This hyperchromic effect likely corresponds to the parallel positioning of three rings in this complex, i.e. the flavin, nicotinamide, and Tyr rings (2). The MDS species has an extinction coefficient at 540 nm almost as high as the thiolate-flavin CT complex of EH$_2$ (7, 28).

The maintenance of a 540 nm band for the EH$_2$ and MDS species of GR suggests no change in the CT interaction between FAD and its thiolate donor. A modification in protein conformation is expected to affect the strength of the CT complex and thus the position of the CT band. This effect is clearly observed when the absorption spectra of the EH$_2$ forms of various pyridine nucleotide-disulfide oxidoreductases are compared. With different protein environments around the FAD and/or the disulfide/dithiol center, the CT band position varies between 500 and 580 nm (32–36).

The x-ray structure of NADH-reduced GR indicates that the CT complex between the $S$(thiolate) of Cys-63 and the $C_{4n}$(FAD) atom is stabilized by the dipole of a well oriented $\alpha$-helix as well as by two hydrogen bonds with the hydroxyl groups of Thr-339 and of the ribityl chain ($O_{2'}$(FAD)) (4, 5). The imidazolium group of His-467 was also proposed to be involved in the transfer of reducing equivalents from the reduced flavin to the disulfide center (4). The crystal structure of the GSH-reduced enzyme gives no indication of a significant change in the distances between the $S$(thiolate) atom and its plausible partners (4). Therefore, the different RR spectra observed for the EH$_2$ and MDS states of GR may be interpreted in the frame of a modified intramolecular CT complex between FAD and $S$(thiolate). It is important to note that the available absorption and crystallographic data are hardly compatible with this interpretation.

Hydrogen Bonding States of the $N_5$(FAD) Site—Except for proton movements, the x-ray structures of GR yield a geometric picture of the catalytic cycle (2–5). Electrons flow from NADPH to GSSG via the isoalloxazine ring and the redox active disulfide without a major conformational change of the protein. The GSH- and NADPH-binding pockets are physically well separated in the GR active site, with the FAD and the redox-active disulfide bridge lying in between (2). Considering this structural arrangement, the mechanisms by which the EH$_2$ and MDS species are formed differ. The $E_{\text{red}}$ reduction with NADPH is mediated by FAD, which is transiently reduced (6, 37). In this case, the electron and proton transfers are oriented from the re-side to the si-side of the flavin (4). On the contrary, the GR reduction by GSH likely proceeds via the si-side by direct opening of the disulfide center to form a dithiol site and subsequently MDSs (7). The different frequencies of bands II and III indicate various degrees of hydrogen bonding at $N_5$ and/or $N_5$ for the MDS form as well as for the two EH$_2$ forms (19). Therefore, in influencing the electrostatic environment of the $N_5$ atom(s) of FAD, GR would discriminate between the two pathways by which the disulfide center was reduced.

The crystal structures of GR show that the isoalloxazine ring of FAD is buried in a deep pocket of the protein, out of the direct influence of solvent molecules (2, 5). The $N_1$ atom has practically no interaction with the protein or the solvent. A distance of 3.49 Å between $N_5$(FAD) and the NH(peptide) group of a conserved Thr residue only suggests the formation of a very weak hydrogen bond (4, 5). On the contrary, the environment of the $N_5$(FAD) atom is relatively polar. The ammonium group (NZ) of a conserved lysine residue (Lys-66 and Lys-50 in human and E. coli GR, respectively) is at a distance of 2.96–3.01 Å (4, 5). Moreover, a network of hydrogen bonds is probably organized around this NZ(Lys) atom. For oxidized GR and GSH-reduced GR, the carboxylate group of a paired glutamate residue (Glu-201 and Glu-181 in human and E. coli GR, respectively) forms a salt bridge with NZ that also interacts with two water molecules (numbered 500 and 568 in human GR) and the $O_4$(FAD) atom (4, 5). In the GSH-reduced enzyme, NZ(Lys-66) is consequently within hydrogen bond distances with $N_5$(FAD) (3.01 Å), $O_4$(FAD) (2.66 Å), water number 500 (3.03 Å), OE1(Glu-201) (3.23 Å), and OE2(Glu-201) (2.77 Å) (4).

In the EH$_2$-NADPH complex of human GR, the carboxylate group of Glu-201, the NZ(Lys-66) atom, and the $N_5$ and $O_4$ atoms of FAD are very similarly positioned (4). Nevertheless, the amide nitrogen (NN7) of NADH apparently displaces the water molecule (numbered 500) strongly associated with Glu-201 in the GSH-reduced enzyme. In the NADH-reduced enzyme, NZ(Lys-66) can thus interact with $N_5$(FAD) (3.0 Å), $O_4$(FAD) (2.76 Å), OE1(Glu-201) (3.36 Å), and OE2(Glu-201) (2.76 Å). Taking into account these structural data, the nature as well as the strength of the interaction between NZ(Lys) and $N_5$(FAD) are not clear because with a maximum of three protons for the NH$_2$(Lys-66) group, all of the potential hydrogen bonds indicated in the crystal structures cannot be engaged simultaneously. From the different frequencies of bands II and III, it seems possible to relate the different hydrogen bonding states of $N_5$ to different configurations of the terminal NH$_2$(Lys-66) group in the NADPH- and GSH-reduced enzymes. Our RR data are consistent with an increased hydrogen bonding state of the NZ(FAD) atom in passing from NADPH-reduced GR to GSH-reduced GR. A discrete rotation of the ammonium protons could be at the origin of an hydrogen bonding rearrangement around $N_5$ in the EH$_2$ and MDS species. A more diffuse mechanism could, however, be envisaged. In GR reduced by NADPH, the transient formation of reduced FAD induces the $N_5$ protonation (6, 37). The interaction between NZ(Lys-66) and $N_5$ is therefore expected to be strongly modified. Upon reoxidation of FAD, a positive density of charge can be moved from $N_5$ to the NH$_2$(Lys-66) group and then to more distant proton acceptors/donors in the protein. In the absence of transhydrogenase activity, the GR reduction by GSH could result in a different network of hydrogen bonds around NZ and $N_5$.

Finally, it is interesting to note that a conserved Lys-Glu pair occupies a homologous position in the structures of lipoamide dehydrogenase, the ammonium function of Lys being near the O$_1$ and N$_5$ sites of FAD (38, 39). The mutation of the conserved Lys-35 by an Arg residue in E. coli lipoamide dehydrogenase influences the enzyme properties (40). In particular, the redox potential of FAD was raised by +60 mV. In contrast, that of the disulfide/dithiol center was unaffected by the Lys/Arg substitution. Considering the structural homologies around the $N_5$ sites of GR and lipoamide dehydrogenase, these observations clearly reveal the sensitivity of $N_5$ to its electrostatic environment and the absence of redox communication between $N_5$ and the disulfide/dithiol center.

Regulation of the Redox Potential of FAD

The functional role of GR is to reduce GSSG from NADPH (Equation 1). Considering that (i) the product of the reaction,
GSH, also reduces GR and (ii) the high NADPH/NADP⁺ and GSH/GSSG ratios in the cytoplasm of normal cells, GR is present largely in the EH₂ and MDS states (28).

The present investigation on yeast GR reveals that the structure and environment of the N₁₀-C₁₀a and N₅=C₄a bonds of FAD differ in the EH₂ and MDS species. On one hand, we have observed a steric or electronic influence of the protein on N₁₀ when GR is reduced with NADPH. This effect may be correlated with the slight distortion of the isoalloxazine ring in the NADPH-reduced enzyme. The high resolution FAD in the GSH-reduced enzyme being in fact lower than that of the NADPH-reduced enzyme. The redox potential of the flavin ring of GR is indirectly modulated by NADPH and GSH, the intrinsic potential of the redox couple (−320–335 mV) (43–45). An increased hydrogen bonding interaction at N₅ is expected to stabilize the oxidized state of flavin, thus decreasing the intrinsic redox potential of FAD and making its reduction less favorable (46). In other words, different hydrogen bonding states at N₅ therefore suggest that the redox potential of the flavin ring of GR is indirectly modulated by NADPH and GSH, the intrinsic potential of FAD in the GSH-reduced enzyme being in fact lower than that of the NADPH-reduced enzyme. The high resolution structure of GR indicates that FAD can be equatorially protonated at N₅ (3). A strong hydrogen bond at the N₅ atom of the GSH-reduced enzyme may constitute an energetic barrier that could limit a protonation of N₅ for the formation of reduced FAD.

In conclusion, regulation of the enzyme activity plausibly involves the oxidized FAD of EH₂ and MDS and the reduced forms of the substrates and products of the enzyme, i.e., NADPH and GSH. Our investigation shows that the formation of EH₂ by GSH reinforces the hydrogen bonding state of the N₅ atom. This electrostatic effect likely decreases the intrinsic redox potential of FAD and thus can decrease the efficiency of redox transfer between NADPH and oxidized FAD for the initiation of a new catalytic cycle.

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