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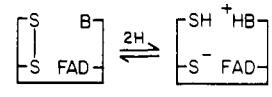
Glutathione Reductase from Yeast. Differential Reactivity of the Nascent Thiols in Two-Electron Reduced Enzyme and Properties of a Monoalkylated Derivative†

L. David Arscott, Colin Thorpe,‡ and Charles H. Williams, Jr.*

ABSTRACT: Two-electron reduced glutathione reductase from yeast reacted with iodoacetamide is alkylated almost exclusively in the nascent thiol nearer the amino terminus of the protein. The charge-transfer absorbance, maximal at 530 nm, characteristic of the two-electron reduced enzyme is not lost as the alkylation proceeds, and the product has a spectrum virtually identical with that of the two-electron reduced enzyme. This observation demonstrates that the thiol alkylated is not the charge-transfer-donor thiolate which interacts with the FAD. The spectrum of the monoalkylated derivative is stable in the presence of oxidized glutathione, indicating that the charge-transfer-donor thiol is not involved in interchange with the substrate in the native enzyme. Thus, the nascent thiols produced upon two-electron reduction of glutathione reductase have distinct functions, interchange with the substrate and interaction with the FAD. Treatment of the monoalkylated derivative with the apolar phenylmercuric acetate eliminates the charge-transfer interaction. The spectrum of the resulting species is similar to that of the oxidized enzyme but less resolved and blue shifted by 10 nm. The dependence on pH of the absorbance associated with the thiolate to FAD charge-transfer interaction in native two-electron reduced glutathione reductase is biphasic, with pK values at approximately 4.8 and 7.4. By analogy with glyceraldehyde-3-phosphate dehydrogenase and papain, these data indicate that the thiolate is stabilized by an adjacent basic residue. The pK 7.4 is associated with the titration of the base to give the ion pair, and the pK of 4.8 is associated with the titration of the thiolate. Unlike lipoamide dehydrogenase, glutathione reductase is sufficiently stable to allow titration with dithionite at pH 3.7. The spectrum at this pH is essentially the same as that of the monoalkylated derivative treated with phenylmercuric acetate. The changes with pH are completely reversible.

Glutathione reductase (EC 1.6.4.2) and lipoamide dehydrogenase (EC 1.6.4.3) are very similar flavoproteins, each catalyzing a specific pyridine nucleotide–disulfide oxidoreduction (Williams, 1976). Both structural and mechanistic similarities have been extensively documented; thus each contains an oxidation–reduction active cystine residue (Searls & Sanadi, 1960a,b; Massey & Veeger, 1960, 1961; Black & Hudson, 1961; Massey & Williams, 1965) which is located in a highly homologous section of the polypeptide chain (Jones & Williams, 1975). Two-electron reduction of either enzyme produces a spectrally characteristic red intermediate (Searls & Sanadi, 1960a,b; Massey & Veeger, 1960, 1961; Black & Hudson, 1961; Massey & Williams, 1965) which is a charge-transfer complex between a thiolate anion (as the donor) and oxidized flavin (as the acceptor) (Searls et al., 1961; Kosower, 1966; Massey & Ghisla, 1974). A base in the active site accepts the second proton (Matthews & Williams, 1976; Matthews et al., 1977). In catalysis, these enzymes cycle between the oxidized (E)[1] and the two-electron reduced (EH_2) states (Massey et al., 1960).



Catalysis by lipoamide dehydrogenase involves a sequential flow of two electron equivalents from dihydrolipoamide to the active center disulfide to FAD to NAD^+ (Searls & Sanadi, 1960a,b; Thorpe & Williams, 1976a,b). In glutathione reductase, this sequence is reversed, beginning with NADPH and ending with thiol–disulfide interchange between the ac-

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[1] Abbreviations used: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; SE-Sephadex, sulfoethyl-Sephadex; E, EH_2, and EH_4 refer to the oxidized, two-electron, and four-electron reduced enzyme species, respectively; EHR, two-electron reduced alkylated derivative; PhHgOAc, phenylmercuric acetate; AAD^+, aminopyridine adenine dinucleotide; thio-NAD^+, thionicotinamide adenine dinucleotide; STI, soybean trypsin inhibitor; GSSG, oxidized glutathione.

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tive-center dithiol and glutathione.

This paper will demonstrate, as has been previously demonstrated for lipoamide dehydrogenase (Thorpe & Williams, 1976a), that the two thiols produced upon two-electron reduction of glutathione reductase are not chemically equivalent. The two nascent thiols in EH_2 are readily distinguished following modification, since the sequences in the region of the active-site disulfide are known for both enzymes (Burleigh & Williams, 1972; Brown & Perham, 1972, 1974; Matthews et al., 1974; Jones & Williams, 1975; Krohne-Ehrich et al., 1977). The properties of monolabeled derivatives of these enzymes give clear indication of the functions of the nascent thiols in catalysis. Thus the thiol nearer the amino terminus seems to be involved in thiol–disulfide interchange while the other thiol interacts with the FAD (Thorpe & Williams, 1976b; Williams et al., 1978). These assignments are supported by the recently reported x-ray crystal structure of erythrocyte glutathione reductase (Schulz et al., 1978).

Materials and Methods

Yeast glutathione reductase purchased from Sigma was further purified as described by Massey & Williams (1965). TPCK-treated trypsin was from Worthington; trypsin from Calbiochem was purified over STI-Sepharose-4B from Pharmacia; α-chymotrypsin was from General Biochemicals. Radioactive iodo[1-^{14}C]acetamide was from Amersham/Searle. Fluram was obtained from Roche Diagnostics. Oxidized enzyme concentrations were expressed with respect to FAD by using an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 462 nm (Massey & Williams, 1965) and in the case of EHR by an extinction coefficient of 3.4 mM^{-1} cm^{-1} at 525 nm as determined by amino acid analysis of spectrally characterized samples.

Spectral Characterization of EHR. Anaerobic titrations with dithionite and other reagents were performed as described by Matthews & Williams (1976) and spectra were recorded on a Cary 118C spectrophotometer as noted by Thorpe & Williams (1976a). The comparison and manipulation of spectra were done as described by Williams et al. (1979).

Assays. Activity measurement using GSSG and NADPH as substrates was done as described by Massey & Williams (1965). In the case where ferricyanide replaced oxidized glutathione, its final concentration was 1 mM. In both cases, the decrease in absorbance at 340 nm was followed at 25 °C and activity was defined as moles of NADPH oxidized per minute per mole of enzyme FAD.

Alkylations of EH_2. Yeast glutathione reductase, 1.07 μmol in 9.4 mL of 0.13 M phosphate buffer, pH 7.21, and 0.3 mM EDTA, was reduced anaerobically with 1.2 equiv of 17.8 mM dithionite and subsequently alkylated in the dark at 25 °C by the addition of 27.7 μmol of iodo[1-^{14}C]acetamide in 0.36 mL of water with a specific activity of 1.44 μCi/μmol. At the end of 2.5 h, the enzyme solution was opened to air and dialyzed at 4 °C for 3 days vs. three changes of 1 L each of 0.1 M phosphate, pH 7.5, and 0.3 mM EDTA.

The remaining thiols were anaerobically alkylated in the dark at 25 °C by adding 95.3 μmol of solid unlabeled iodoacetamide to 0.953 μmol of enzyme FAD in 9.0 mL of 0.1 M phosphate, pH 7.6, and 0.3 mM EDTA. After approximately 45 min, 25 mL of an anaerobic solution of 8.0 M guanidinium chloride in 0.1 M phosphate, pH 7.5, and 0.3 mM EDTA was added to thoroughly denature the enzyme. After 18 h of alkylation, the enzyme solution was dialyzed for 2 days vs. two changes of 500 mL each of 3.0 M guanidinium chloride in 0.1 M phosphate buffer, pH 6.0, and 0.3 mM EDTA, then exhaustively dialyzed vs. 1% ammonium bicarbonate, at the end of which the enzyme was lyophilized prior to proteolytic digestion.

Trypsin Digestion of Alkylated Protein and High-Voltage Electrophoresis. Proteolytic digestion was performed by using either TPCK-treated or STI-Sepharose-4B-purified[2] trypsin in 0.5% ammonium bicarbonate, by the addition of 2% w/w aliquots of trypsin at 0, 1, and 5 h in the first or 0 and 2 h in the second preparation. The digestion was stopped by freezing after 17 h in the first and 5 h in the second. High-voltage paper electrophoresis (in pyridine–acetic acid–water, pH 6.5; 100:4:900) of approximately 1% of the ^{14}C-labeled protein digest monitored the progress of proteolytic digestion. The electrophoresis was carried out on Whatman 3 MM paper, 8 × 68 cm, for 60–75 min at either 3 kV or 4.5 kV with a tank system from Savant Instrument, Inc. The radioactive strips were scanned and areas integrated with a Nuclear Chicago Actigraph III with a Model 8735 digital integrator. The strips were stained when appropriate with either a ninhydrin–cadmium acetate solution (Atfield & Morris, 1961) or Fluram (Jones & Williams, 1975).

Separation of Radioactive Tryptic Peptides. The tryptic digest was applied to a column of DEAE-cellulose (DE52 from Whatman), 1.5 × 90 cm, equilibrated with 20 mM ammonium bicarbonate, and peptides were eluted with a nonlinear gradient, concave upward, from the equilibrating buffer to 1.0 M ammonium bicarbonate. The gradient was generated as previously described (Thorpe & Williams, 1976a). Radioactivity was followed by sampling 0.1–5% of the volume of various fractions where necessary and counting as described by Jones & Williams (1975). Appropriate radioactive fractions were pooled, lyophilized, and applied to a column of SE-Sephadex, 0.9 × 52 cm, equilibrated with 0.05 M pyridine–acetate buffer, pH 2.5. Elution was accomplished by using a concave-upward gradient from equilibrating buffer to 2.0 M pyridine–acetate buffer, pH 5.0.

α-Chymotrypsin Digestion of a 14-Residue Cationic Peptide. The ^{14}C-labeled peptide from the previous SE-Sephadex column was lyophilized and redissolved in 0.5 mL of 1% ammonium bicarbonate. Proteolytic digestion at 37 °C was started by the addition of 6% w/w of α-chymotrypsin with a subsequent addition of 4% w/w after 2 h and stopped after 7 h by freezing; the peptide was then lyophilized. Analytical high-voltage electrophoresis on less than 1% of the solution monitored the progress of digestion.

Separation and Identification of Peptide Fragments. Separation of the digestion products was accomplished by redissolving the lyophilized material in 0.05 M pyridine–acetate buffer, pH 2.5, and applying it to a 0.9 × 52 cm SE-Sephadex column equilibrated with the same buffer. A linear gradient from equilibrating buffer to 0.64 M buffer, pH 2.5, resolved major peptides, with additional purification being accomplished via preparative high-voltage electrophoresis in acetic acid–formic acid–water buffer, pH 1.9 (1:10:89). The radioactive areas were cut out of the electropherograms and allowed to soak in 10% v/v acetic acid for 1–2 h. The acetic acid solution was then dried quickly on a flash evaporator. The yield using this method was generally in the range 60–85%.

Results

Reduction and Monoalkylation. The reduction of glutathione reductase with 1 equiv of sodium dithionite generates the 2-electron reduced enzyme, EH_2. This species (Figure 1) with its characteristic long-wavelength band is a charge-

[2] B. D. Burleigh, personal communication.

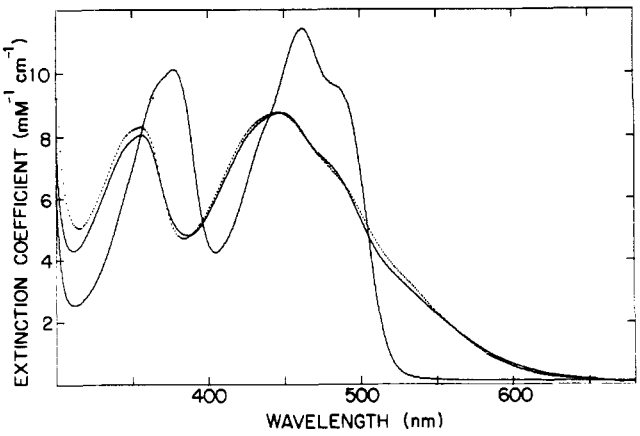


FIGURE 1: Spectra of yeast glutathione reductase, oxidized, two-electron reduced, and two-electron reduced after alkylation. The protein, 1.07 μmol (enzyme-bound FAD), in 9.4 mL of 0.13 M phosphate, pH 7.21, and 0.3 mM EDTA, was reduced anaerobically with 1.2 molar equiv of $Na_2S_2O_4$. Dotted curve, 2.5 h after the addition of 27.7 μmol of iodoacetamide at 25 °C.

Table I: Activity Measurements of Native and Monoalkylated Glutathione Reductase

	native	monoalkylated
NADPH/GSSG turnover[a]	14810	346
NADPH/$Fe(CN)_6^{3-}$ turnover[a]	713	345
NADPH/thio-$NADP^+$ turnover[b]	170	20
K_i(NADPH)[c]	2 μM	18-30 μM
NADH/thio-$NADP^+$ turnover[b]	145[d]	4.7
K_i(thio-$NADP^+$)[e]	87 μM[d]	74 μM

[a] Units are moles of NADPH oxidized per minute per mole of enzyme FAD. [b] Units are moles of thio-$NADP^+$ reduced per minute per mole of enzyme FAD. [c] NADPH/thio-$NADP^+$. [d] From the data of Moroff et al. (1976). [e] NADH/thio-$NADP^+$.

transfer complex. The addition of excess [^{14}C]iodoacetamide produced such minor spectral changes (Figure 1) that further chemical studies might not have been attempted in light of observations with pig heart lipoamide dehydrogenase (Thorpe & Williams 1976b, 1981). In that alkylation reaction, the spectrum of the two-electron reduced intermediate changed to that of the oxidized enzyme, slightly blue shifted and less resolved. That glutathione reductase had been alkylated was indicated by the failure of either ferricyanide or oxidized glutathione to reoxidize the enzyme. Amino acid analysis showed 1.1 mol of *S*-(carboxymethyl)cysteine per FAD with a specific activity equal to that of the reagent (2827 dpm/nmol of Cys(CM)). No other amino acids were modified. Samples were withdrawn during the reaction for activity measurements; inactivation was half-complete in 20 min, and roughly 2% of the native activity remained at the end of alkylation. On the other hand, the NADPH/ferricyanide assay showed only a 50% decrease in activity (Table I). The transhydrogenase activity was markedly inhibited by the monoalkylation, in contrast to the analogous finding with lipoamide dehydrogenase.[3]

Massey & Williams (1965) had shown that prior reduction of the enzyme was not necessary to titrate one sulfhydryl group with phenylmercuric acetate at pH 9.0. It was important therefore to show if this sulfhydryl group would react with iodoacetamide under conditions similar to those used in the

[3] J. R. Babson and D. J. Reed, Department of Biochemistry and Biophysics, Oregon State University, have also found marked inhibition of the transhydrogenase in gluthathione reductase EH_2 treated with isocyanates (personal communication).

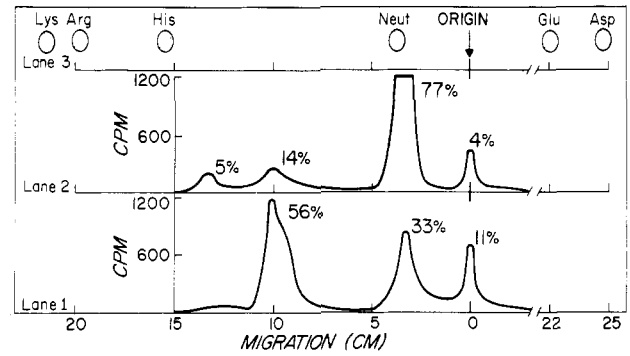


FIGURE 2: High-voltage electropherograms of a tryptic and subsequent chymotryptic digest of alkylated glutathione reductase. Lane 1 is the profile of radioactivity from 10 nmol of alkylated enzyme after 17 h of TPCK-trypsin digestion. Lane 2 is the profile of 16 nmol of the same digest after an additional 2 h of digestion with 2% w/w of α-chymotrypsin. Lane 3 shows the ninhydrin-positive spots from 25 nmol each of a standard mixture of 18 amino acids used as mobility markers.

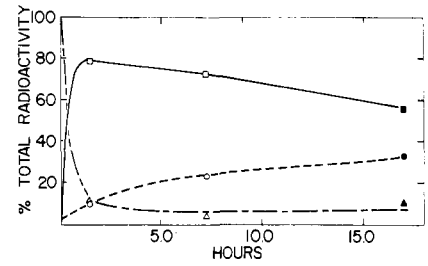


FIGURE 3: Yield of the three radioactive spots observed on electropherograms (as seen in Figure 2, Lane 1) vs. time of tryptic digestion of alkylated yeast glutathione reductase. A 14-residue cationic peptide (□), neutral peptide (○), and undigested or "core" material (△). TPCK-trypsin (solid symbols) was used in the first digestion of EHR and trypsin purified via an affinity column (open symbols) was used for the second preparation (see Materials and Methods).

alkylation of EH_2. Oxidized enzyme (45 μM) was reacted for 3 h at pH 6.5 with a 16-fold excess of iodo[1-^{14}C]acetamide. No detectable radioactivity was bound to the enzyme. Thus it appeared that reduction was a necessary requisite for monoalkylation under these conditions. However, a conformational change upon reduction with consequent exposure of a previously unreactive cysteine residue could not be ruled out. Thus, the isolation and purification of the redox-active disulfide peptide were carried out to determine which of the active-center half-cystines was radiolabeled.

Peptide Isolation and Characterization. The monoalkylated enzyme is referred to as EHR by analogy with the similar lipoamide dehydrogenase derivative in which the deprotonation of EH_2 upon alkylation has been demonstrated. EHR was further alkylated with cold iodoacetamide in 5.6 M guanidinium chloride. Of the remaining five cysteines, an average of 3.7 sulfhydryl groups was alkylated in two large-scale preparations. Trypsin digestion of the alkylated protein with TPCK-treated trypsin gave the expected mobility on analytical high-voltage electrophoresis for a 14-residue cationic peptide with one positive charge (Figure 2, lane 1) (Jones & Williams, 1975). The low yield of this peptide (56% after 17 h of digestion) was improved to 75% in a second large-scale preparation of EHR by digestion for only 7.5 h with trypsin purified by passage over an affinity column of Sepharose-4B to which soybean trypsin inhibitor had been attached. It was felt that some contaminating proteolytic enzyme(s) in the TPCK-treated trypsin might be responsible for the hydrolysis between the two active-center cysteines, giving rise to the neutral peptide as seen in Figure 2, lane 1. The data in Figure 3

Table II: Amino Acid Composition, Charge, and Mobilities of Tryptic and Chymotryptic Active-Site Peptides

amino acid	tryptic disulfide peptide	chymotryptic products[a]				
		1	2A	2B	(3 + 4)A	(3 + 4)B
Lys	1.4				0.9	0.9
Cys(CM)	1.8	0.8	1.1	1.1	0.9	0.9
Asp	1.0[d]	1.0[d]	0.9	0.4	1.0[d]	
Thr	1.0	1.0	1.0	1.0		
Pro	1.1				1.0	1.1
Gly	3.0	2.2	2.1	2.1	1.1	1.0[d]
Ala	1.1	0.9	1.0[d]	0.9		
Val	2.8	1.1	0.9	0.4	2.6	1.7
Leu	1.1	1.0	1.1	1.0[d]		
dpm/nmol (Cys(CM))	1630	2852	2826	3001	360	268
no. of residues	14	8	8	6	8	6
charge at pH 6.5[b]	+1	0	0	0	+1	+1
mobility[c]			0.26	0.32	0.57	0.67

[a] Numbers refer to the peaks seen in Figure 4 and letters to the spots eluted after high-voltage electrophoresis at pH 1.9. [b] Determined by high-voltage electrophoresis (Offord, 1966). [c] Ratio of spot migration to that of lysine by high-voltage electrophoresis at pH 1.9. [d] Residue that is used to calculate molar equivalents.

indicated that the increased yield of this 14-residue peptide was more a function of shorter digestion times than of the purity of trypsin. Thus, the shorter time in the second digestion gave a decreased yield of the neutral peptide which was the result of secondary hydrolysis between the half-cystines (as shown below) either by some contaminating proteolytic enzyme(s) in both preparations of trypsin or inherent chymotrypic-like activity of the trypsin (Plapp et al., 1967). The frank addition of α-chymotrypsin, 2% w/w, to a small portion of the first tryptic digest caused a further rapid loss of the cationic radioactive spot to a neutral peptide with a 77% yield as seen in lane 2 of Figure 2. Chymotryptic digestion of the purified, radioactive, 14-residue cationic peptide was undertaken in order to determine the exact position of the radiolabel.

The tryptic digest was applied to a DE52 column from which 72% of the radioactivity emerged in the void volume. This was lyophilized and further purified on an SE-Sephadex column. A ninhydrin-positive peak emerging at the 0.5 M position in the buffer gradient contained 85% of the radioactivity. Amino acid analysis (Table II) showed those residues expected for the tryptic disulfide peptide. The specific activity of this peptide was approximately half that of the iodo[1-^{14}C]acetamide used. An analytical high-voltage electropherogram of this fraction showed it to be identical in mobility to the cationic peak seen in lane 1 of Figure 2. A second radioactive peak emerging just after the void volume contained 12% of the radioactivity. Amino acid analysis and electrophoresis of this peak showed that it was an 8-residue neutral peptide representing the amino-terminal half of the 14-residue tryptic disulfide peptide and arising from secondary splitting during digestion (see above). The specific activity of this peptide was the same as that of the iodo[1-^{14}C]acetamide used.

The purified tryptic peptide was digested with α-chymotrypsin and the fragments were purified by SE-Sephadex chromatography, as seen in Figure 4. Peaks 2 and 3 plus 4 were further purified by high-voltage electrophoresis at pH 1.9 (see Materials and Methods). The compositions of the chymotryptic peptides obtained are shown in Table II. These results showed that the cysteine residue closest to the amino terminus of the active-site peptide was the primary reactant in the monoalkylation, while the cysteine residue closest to carboxyl terminus was almost 10 times less reactive [(3 + 4)A and (3 + 4)B]. Figure 5 shows the sequence of the disulfide peptide (Jones & Williams, 1975) and the cleavage points by chymotrypsin (Table II). Inspection of Table II shows that both peak 1 and spot A from peak 2 gave identical amino acid analyses. The reason for the differential mobility on SE-

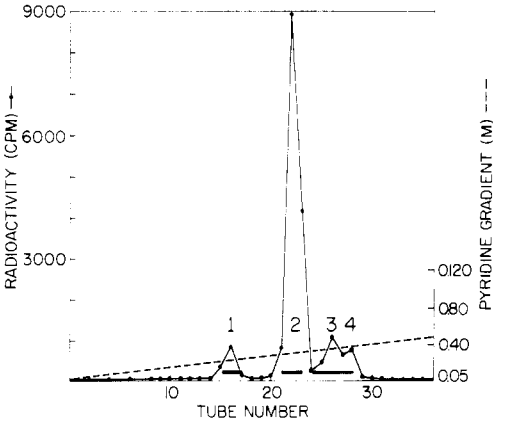


FIGURE 4: Chromatography on SE-Sephadex of the 7-h chymotryptic digest of the 14-residue cationic tryptic active-site peptide. Tubes were combined into three main fractions for subsequent analysis, as indicated by the bars. Peak 2 and peaks 3 + 4 were subjected to additional purification via preparative high-voltage electrophoresis.

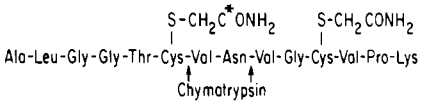


FIGURE 5: Sequence of the tryptic active-site peptide. Arrows show points of hydrolysis by chymotrypsin.

Sephadex is unclear since both peptides gave identical mobilities on high-voltage electrophoresis, thus eliminating the possibility of oxidation of Cys(CM) or the hydrolysis of the asparagine to aspartic acid.

Spectral Experiments. EHR is a very stable derivative. A solution stored at 4 °C in the dark at pH 7.6 shows only minor spectral changes over a period of 1 year. As noted previously, the long-wavelength absorption band of EHR was not discharged on treatment with $Fe(CN)_6^{3-}$ or GSSG, two oxidants which readily reoxidize EH_2.

The aerobic addition of up to 142 μM $NADP^+$ to 16 μM EHR at pH 6.3 produced small increases in long-wavelength extinction beyond 550 nm (ϵ_{600} from 0.65 mM^{-1} cm^{-1} to 1.00 mM^{-1} cm^{-1}). These changes were similar in form (but of lesser magnitude) to those observed with EH_2-$NADP^+$ complexes (Williams et al., 1976). This behavior was in marked contrast to that observed on the addition of NAD^+ to EHR from lipoamide dehydrogenase (Thorpe & Williams, 1976b) (see Discussion).

In contrast to the spectral stability of EHR to the oxidants, phenylmercuric acetate caused the loss of the charge-transfer

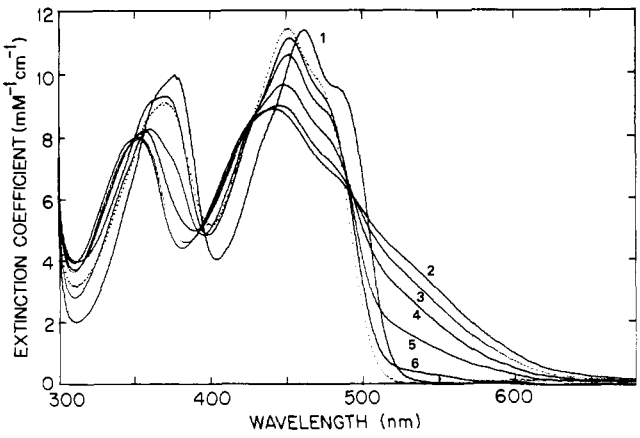


FIGURE 6: Spectra of yeast glutathione reductase, E, and EH_2 at different pH values, and EHR in the presence of PhHgOAc. Curve 1, oxidized enzyme at pH 7.6; curves 2, 3, 4, 5 and 6 are spectra extrapolated to full formation of EH_2 at pH 8.1, 7.1, 5.4, 4.7, and 3.6, respectively. The dotted curve is an extrapolated spectrum of EHR in the presence of PhHgOAc at pH 7.6 (see text).

band centered around 540 nm with a simultaneous increase at both 452 and 380 nm (dotted curve, Figure 6). This curve was constructed [see Williams et al. (1979) and Materials and Methods] from an observed spectrum at 1.8 PhHgOAc/FAD (corresponding to 82% reduction in long-wavelength absorbance) by using an extrapolated end point of 2.2 PhHgOAc/FAD for complete loss of the long-wavelength band. Following 1.8 equiv of PhHgOAc/FAD, excess β-mercaptoethanol rapidly regenerated the spectrum of EHR.

Very similar spectral changes to those induced by mercurial on EHR were produced on protonation of the two-electron reduced native enzyme. The spectra of fully formed EH_2 at several pH values are shown in Figure 6. Each spectrum was obtained from a separate dithionite titration. Again, a decrease was seen in the charge-transfer band at 540 nm as the pH was lowered with a concomitant rise at 452 and 380 nm. Isobestic points were observed at 492 and 394 nm. A plot (Figure 7) of the pH dependence of the $\Delta\epsilon_{540}$ was biphasic, with indicated pK values of 4.8 and 7.4. The reversibility of the pH effect was demonstrated when the species seen in the pH 3.6 experiment (Figure 6, curve 6) was raised to pH 5.1 with the reappearance of the spectrum expected at that pH. Yeast gluthatione reductase was surprisingly stable at pH 3.6, and what little precipitate was observed was centrifuged away before anaerobiosis was established. The resulting oxidized anaerobic spectrum (not shown) when compared to those obtained at the higher pH values showed very small changes. The most notable was a diminution in the 480-nm shoulder of approximately 0.5 mM^{-1} cm^{-1}, suggestive of a pH effect rather than release of FAD. The visible spectral ratios 462 nm:406 nm and 462 nm:378 nm of the oxidized enzyme at the different pH values were also not changed, giving further evidence that the FAD was still in a substantially native configuration. Preliminary studies indicate that the long-wavelength band of EHR, like that of EH_2, is pH dependent.

Discussion

The results presented in this paper have described the preparation of a monoalkylated derivative of two-electron reduced glutathione reductase and have shown that the cysteine residue closest to the amino terminus in the active-site peptide is at least 8-fold more reactive than its redox partner toward iodoacetamide. The derivative retains a catalytically competent pyridine nucleotide binding site, since NADPH binds tightly to EHR (Table I), inducing spectral changes similar

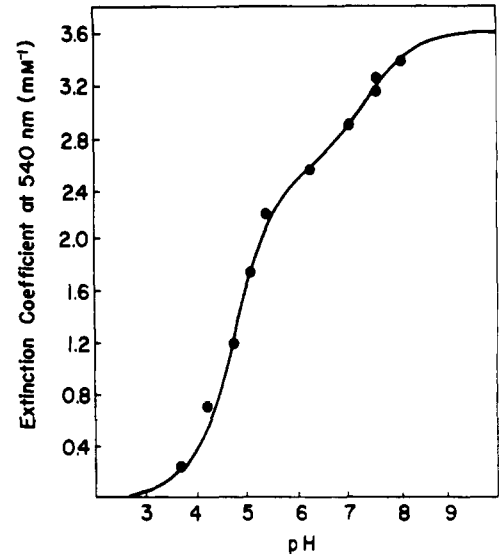
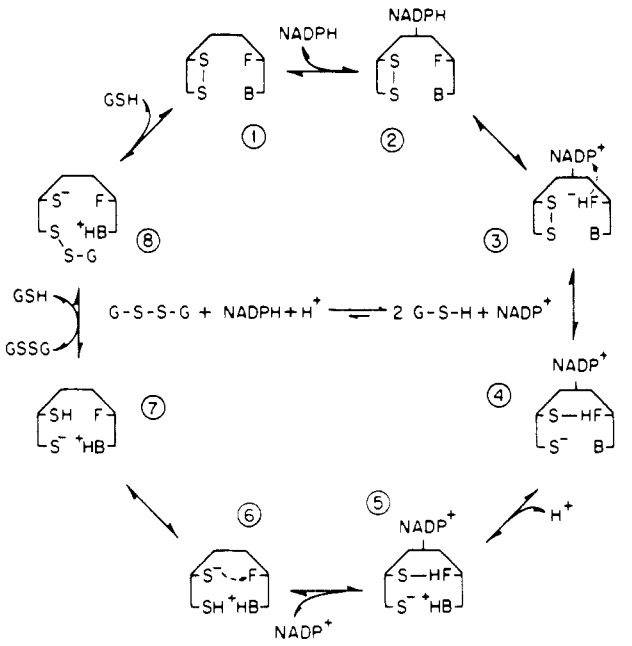


FIGURE 7: Dependence of 540-nm absorbance of yeast glutathione reductase EH_2 on pH. The difference extinction coefficients ($\Delta\epsilon$) plotted are obtained by subtracting the extrapolated ϵ_{540} values for EH_2 from the comparable value for oxidized enzyme at the same pH. Oxidized enzyme (30–40 μM) was titrated anaerobically with dithionite. The buffer was 85 mM citrate and 0.3 mM EDTA at pH 3.7, 4.2, 4.8, 5.1, and 5.4, while at pH 6.3, 7.1, 7.6, and 8.1 100 mM phosphate replaced the citrate. The solid line assumes pK values of 4.8 and 7.4 and extinctions of 0, 2600 and 3600 M^{-1} cm^{-1}. At pH values of 7.1, 7.6, and 8.1, a concentration of methyl viologen equal to 1% of the flavin concentration was used.

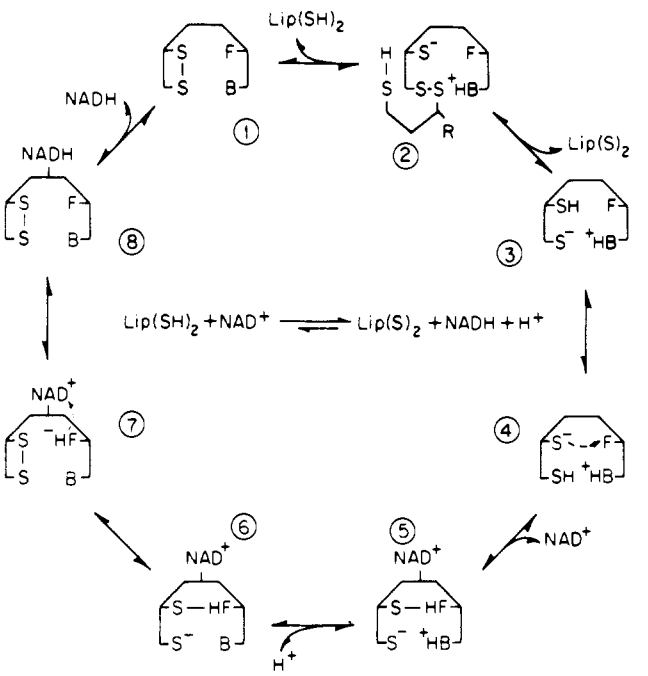
to those seen in EH_2 [Figure 5 in Williams et al. (1976)]. Further, EHR exhibits approximately 50% of the activity of the native protein in the NADPH/ferricyanide assay, indicating that NADPH is still capable of transferring reducing equivalents to ferricyanide via the bound flavin. In contrast, the derivative is essentially unable to catalyze the NADPH-dependent reduction of GSSG. These results are analogous to those obtained earlier by use of pig heart lipoamide dehydrogenase EHR and provide additional evidence for a sequential flow of reducing equivalents from NAD(P)H to flavin to active–site disulfide bridge to disulfide substrates in these enzymes (Searls & Sanadi, 1960a,b; Thorpe & Williams, 1976a,b). Due to severe substrate inhibition by NADPH in glutathione reductase, it is very difficult to measure the transhydrogenase activity of EHR (or EH_2) as was done with lipoamide dehydrogenase EHR. The minimal diminution of the NADPH–ferricyanide activity of glutathione reductase EHR indicates that the ferricyanide can interact with the flavin in the EHR–NADPH complex whereas oxidized pyridine nucleotide analogues do not compete effectively with NADPH.

The long-wavelength band of two-electron reduced glutathione reductase and lipoamide dehydrogenase is believed to reflect a charge-transfer interaction between thiolate as the donor and oxidized flavin as the acceptor (Kosower, 1966; Massey & Ghisla, 1974). The close spectral similarity between EH_2 and EHR forms of glutathione reductase (Figure 1) allow us to designate the carboxyl-terminal cysteine residue as the charge-transfer donor, since its redox partner is alkylated in EHR. The disappearance of the long-wavelength band of EH_2 at low pH (Figures 6 and 7) or on the addition of mercurial to EHR (Figure 6) provides further evidence of the role of a thiolate in this charge-transfer complex. It should be noted that EHR treated with mercurials and EH_2 at pH 3.6 show very similar spectra, in which loss of the 540 nm band is accompanied by the reappearance of a spectrum of oxidized bound flavin 10 nm blue shifted from the parent native oxidized enzyme. Similar blue shifts are observed during con-

Scheme I

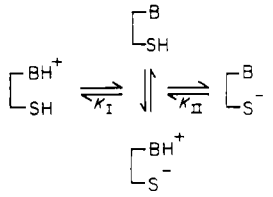


Scheme II



version of pig heart lipoamide dehydrogenase to its EHR form and *Escherichia coli* lipoamide dehydrogenase to a fluorescent non-charge-transfer form of EH_2 (Wilkinson & Williams, 1979). These spectral similarities suggest that cleavage of the disulfide bridge per se induces similar changes in conformation or polarity within the active site of these enzyme forms. Similar shifts of 10 nm were observed in the spectra of the blue semiquinone of thioredoxin reductase upon reduction of the disulfide (Zanetti et al., 1968).

The pH dependence of the charge-transfer absorbance clearly reflects 2 pK values (Figure 7). We have fit the data satisfactorily using pK values of 7.4 and 4.8. These data are very reminiscent of those of Polgar (1975) with papain and glyceraldehyde-3-P dehydrogenase. In those enzymes an ion pair of cysteine and histidine residues is the major species present in the active center at neutral pH values. Indeed, it has been shown in papain that the central equilibrium shown below lies about 10:1 in favor of the ion pair (Lewis et al., 1976).

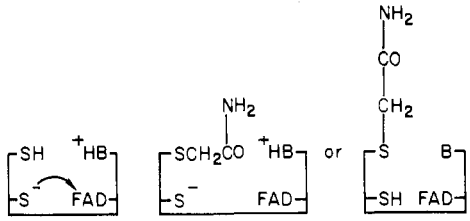


Thus, the macroscopic pK_I is associated primarily with the protonation/deprotonation of the thiol and pK_{II} with the imidazole. pK_I and pK_{II} have values of 4.2 and 7.6, respectively, in papain (Lewis et al., 1976). Lipoamide dehydrogenase is known to have a base at the active site which deprotonates the dithiol substrate and then in its protonated form stabilizes the thiolate charge-transfer donor (Matthews & Williams, 1976). Matthews et al. (1977) found a pK of 7.9 associated with both the release of protons on reduction of the enzyme and the reactivity of EH_2 with iodoacetamide. The three experiments are probably measuring the ionization of the same group in the enzyme which, by analogy with glutathione reductase, we assume to be a histidine residue (Untucht-Grau et al., 1979).

A large body of evidence establishes the close structural and mechanistic similarities between lipoamide dehydrogenase and glutathione reductase outlined in the introduction. Analogous outlines of the mechanisms are given in Schemes I and II. They derive from earlier proposals of Sanadi (1963) and of Massey, (1963). We use them as working hypotheses. The physiological direction of catalysis is depicted as clockwise in both cases. Referring to Scheme 1 for glutathione reductase, EH_2 is an equilibrium mixture of species 6 and 7, and species 6 is the charge-transfer complex. The rate-limiting step is in the second half reaction; all the preceding steps are very fast and are probably concerted. The covalent bond in species 4 and 5 is between the sulfur and the C(4a) of the isoalloxazine by analogy with lipoamide dehydrogenase (Thorpe & Williams, 1981). The mechanism infers that the two catalytically essential thiols produced upon reduction play different roles in catalysis. Thus, we have represented the "upper" thiol as participating in charge transfer and covalent adduct formation with FAD whereas the "lower" thiol interacts with the substrate, glutathione, through a mixed disulfide. The data presented in this paper show that the electron-transfer thiol is nearer the carboxyl terminus while the interchange thiol is nearer the amino terminus in the primary sequence.

The active-site disulfide peptide was isolated from the radioactive monoalkylated glutathione reductase (EHR) which subsequently had been exhaustively alkylated in the presence of guanidinium chloride with unlabeled iodoacetamide. This 14-residue peptide, having half the specific activity of the reagent, was digested with chymotrypsin as had been done in analogous experiments with lipoamide dehydrogenase (Thorpe & Williams, 1976a). However, unlike the active-center peptide from lipoamide dehydrogenase which gave a single pair of peptides, the glutathione reductase peptide gave at least two pairs (Table II, columns 4–7). These data indicate that there are two cleavage points, one between Asn and Val and another between Cys and Val (Figure 5). The variable yields obtained in eluting these peptides from paper make relative quantitation tenuous. The first of these cleavage points is the same as that observed as a side reaction in the tryptic digestions. In any case, these data allow an unambiguous assignment of the position of monoalkylation of EH_2. The neutral peptides emanating from the portion of the tryptic peptide closest to the amino terminus of the protein have specific activities at least 8-fold higher than the cationic peptides arising from the

Scheme III



carboxyl-terminal portion. Thus, the reactive thiol in glutathione reductase EH_2 is the same as that found in lipoamide dehydrogenase (Thorpe & Williams, 1976a). It is highly probable that structurally equivalent redox-active cysteines perform equivalent functions in these two enzymes, particularly since the disulfide regions are so highly homologous (Jones & Williams, 1975).

Two conspicuous differences in behavior between EHR derivatives of glutathione reductase and lipoamide dehydrogenase have assisted in the assignment of roles to the two halves of the active-center cystine residue. The first concerns the lack of long-wavelength absorption in EHR from lipoamide dehydrogenase. We conclude in this case that alkylation of the amino-terminal position sufficiently perturbs the environment of the charge-transfer donor to disturb its orientation toward the FAD or to increase its microscopic p*K*. These two possibly interrelated effects are depicted in Scheme III. It should be noted that the active site of lipoamide dehydrogenase accommodates a relatively small hydrophobic substrate, dihydrolipoamide, whereas glutathione reductase must bind the large polar hexapeptide, oxidized glutathione. Thus, disruption of the active-center region by the somewhat polar carboxamidomethyl label may well be slight with glutathione reductase, allowing retention of the charge-transfer absorption, but more serious in lipoamide dehydrogenase, causing the return of the oxidized flavin spectrum. However, a long-wavelength band is observed in EHR from heart lipoamide dehydrogenase on the addition of AAD^+, and this is most reasonably assigned to a charge-transfer complex between the carboxyl-terminal S moiety and FAD which is recovered on binding this pyridine nucleotide (Thorpe & Williams, 1981).

A second difference in behavior between the two EHR derivatives concerns their response to oxidized pyridine nucleotide. Very minor spectral changes are observed when $NADP^+$ is added to glutathione reductase EHR, whereas NAD^+ induces extensive bleaching of the flavin chromophore of lipoamide dehydrogenase EHR, suggestive of a covalent adduct between the carboxyl-terminal S moiety and the C-4a position of the isoalloxazine ring (Thorpe & Williams, 1981). The reason for this difference is not clear, but it may be related to the fact that physiologically glutathione reductase oxidizes pyridine nucleotide while lipoamide dehydrogenase reduces pyridine nucleotide. Thus, a lesser tendency for electron flow from thiol to FAD might be expected in glutathione reductase. Indeed, recent results of Matthews et al. (1979) imply that the binding of oxidized pyridine nucleotide to lipoamide dehydrogenase EH_2 should influence the distribution of electrons between flavin and dithiol so as to increase the amount of reduced flavin. The behavior of glutathione reductase EHR closely resembles that of the native EH_2 forms of both enzymes since the characteristic spectral features expected for 4a adduct formation are not observed in the EHR–$NADP^+$ complex of glutathione reductase or in the EH_2–NAD^+ complex of lipoamide dehydrogenase. These complexes are characterized by decreased absorbance in the 530-nm region and increased absorbance in the 580-nm region relative to EH_2 (Veeger & Massey, 1963; Matthews et al., 1976; Williams et al., 1976). Nevertheless, all the evidence to date suggests that the covalent adduct induced by NAD^+ is a likely catalytic intermediate in lipoamide dehydrogenase (Thorpe & Williams, 1981).

These conclusions have received powerful independent support from the recently published three-dimensional structure of glutathione reductase from human erythrocytes at 3-Å resolution (Pai et al., 1978; Schulz et al., 1978). The three-dimensional structure of the abortive oxidized enzyme–GSSG complex suggests that the amino-terminal cysteine residue participates in mixed disulfide bond formation with glutathione (Schulz et al., 1978). Studies from this laboratory, performed at the two-electron reduced level of glutathione reductase and lipoamide dehydrogenase, are also consistent with this role. Incubation of glutathione reductase EHR with high concentrations of GSSG fails to abolish the charge-transfer band, suggesting that the carboxyl-terminal thiolate is not the nucleophile which initiates mixed disulfide formation. In addition, the pH dependence of the rate of reoxidation of lipoamide dehydrogenase EH_2 by $LipS_2NH_2$ (the rate-limiting half-reaction) is very similar to the rate of alkylation with iodoacetamide (Matthews et al., 1977), but clearly different from the pH dependence of the charge-transfer absorption band (Matthews & Williams, 1976). These observations argue that of the two redox active cysteines, the amino-terminal one is the most effective nucleophile toward external reagents. We have tried without success to obtain stable derivatives of these enzymes which are modified solely on the carboxyl-terminal position, hoping to demonstrate directly mixed disulfide formation with their substrates.

Acknowledgments

We are grateful for the skilled technical assistance of Mr. Willie Horton and Mr. Erich Phillips. Dr. Frederick A. Johnson carried out one of the experiments at pH 7.6 in Figure 7 and suggested the use of catalytic quantities of methyl viologen to catalyze the reduction at high pH, making the investigation in this region feasible.

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Pressure-Induced Inactivation of Sarcoplasmic Reticulum Adenosine Triphosphatase during High-Speed Centrifugation†

Philippe Champeil,* Sylvie Büschlen, and Florent Guillain

ABSTRACT: Sarcoplasmic reticulum vesicles were found to be highly sensitive to high-speed centrifugation in metal-deprived mediums at low temperature (4 °C). The irreversible modifications induced were easily detected from observation of the environment-sensitive spectrum of an iodoacetamide spin-label bound to the ATPase. Centrifugation also resulted in vesicle aggregation and inhibition of calcium transport, ATPase activity, and phosphoenzyme formation. These denaturation-like phenomena were prevented in the presence of sucrose, or by nucleotide binding, or, again, by cation binding to the ATPase high-affinity calcium binding sites and were only present when centrifugation was performed at low temperature. The crucial parameter during this process was found to be the hydrostatic pressure which developed in the centrifuge tube. SR vesicles exposed to 800 bars in a pressure bomb displayed the same features. It is suggested that irreversible denaturation takes place after one or both of the two following well-documented effects of pressure: a rise in the lipid order/disorder transition temperature or dissociation of the oligomeric structure of the calcium pump.

Although high hydrostatic pressure affects the structure and function of biological systems in many ways, little attention is generally paid to the pressure conditions prevailing during the centrifugation steps included in routine experimental procedures. A number of reports, however, point to the importance of this parameter [see, for instance, Wattiaux-De-Conninck et al. (1977); Formisano et al. (1978)]. In the course of our own spin-labeling studies with sarcoplasmic reticulum (SR)[1] vesicles, we found that the final bound label spectrum was dependent on the conditions prevailing during the free-label-removing centrifugation steps. The resulting enzymic

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[1] Abbreviations used: ATPase, adenosine triphosphatase; SR, sarcoplasmic reticulum; Mes, 2-[*N*-morpholino]ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, morpholinopropanesulfonic acid; SH, sulfhydryl group; ESR, electron spin resonance; ISL, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide (Syva); EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate); ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/81/0420-1520$01.25/0