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STUDIES ON THE ACTIVE SITE OF HUMAN ERYTHROCYTE GLUTATHIONE REDUCTASE USING 6-SCN-FAD AND 6-MERCAPTO-FAD

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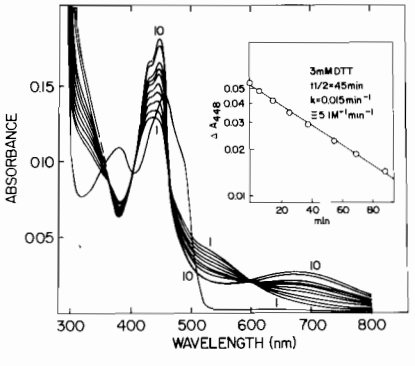
Glutathione reductase (GR) is a member of a group of mechanistically similar flavoproteins, which all contain a redox-active disulfide in addition to FAD. Because its structure has been solved by X-ray diffraction analysis (1,10), GR from human erythrocytes has become the prototype of this group of enzymes, especially in view of the extensive spectral and sequence homologies found within this group (2,3). Such homologies have been used to identify successfully the catalytically essential disulfide of mercuric reductase and in the production of a series of active site mutants of that enzyme (4). Despite the impressive similarities, there do exist, however, significant structural differences in the relative orientation of the flavin and active site disulfide, as will be shown in this and an accompanying communication (5). This was made possible by replacing the native FAD with the chemically reactive flavins, 6-SCN-FAD and 6-mercapto-FAD (6).

Preparation of Modified Holoenzymes

The apoprotein of GR was prepared by the method of Fritsch as described in Manstein et al. (7); DTT, however, was not included in the final solution. The apoprotein was dissolved in 0.05 M phosphate, pH 7.0, containing 0.3 mM EDTA, and was added in slight excess to a solution of 6-SCN-FAD or 6-mercapto-FAD in the same buffer. In general the mixture was incubated in ice for 20 min followed by incubation at 20° for 10 min. The resulting holoenzyme was either used as such, or concentrated by centrifuging through an Amicon Centricon 30 Ultrafilter. In the case of preparation of the 6-mercapto FAD enzyme for crystal studies, 6-mercapto FAD was added in slight excess over apoprotein; after reconstitution the excess flavin was removed by Centricon ultrafiltration.

Conversion of 6-SCN-FAD Enzyme to 6-Mercapto-FAD Enzyme

6-Thiocyanato-FAD binds tightly to the apoprotein of GR and is very stable in the absence of added thiols. The holoenzyme has λ_{max} of 456 nm (compared to 446 nm for free 6-SCN-FAD) and a well resolved maximum at 380 nm (compared to a shoulder with the free flavin). On addition of DTT there was an immediate color change from yellow to red, with a spectral change (Figure 1, curve 1) typical of the EH_2-form of the native enzyme, i.e. a charge transfer complex involving Cys 63 of the reduced active site disulfide as donor and oxidized flavin as acceptor (8). This is followed by a secondary isosbestic reaction in which the flavin is converted to 6-mercapto-FAD. The final spectrum shown (curve 10) is identical to that obtained by titration of 6-mercapto-FAD (λ_{max} 443 nm, ε = 18900 M^{-1} cm^{-1}) with apo-GR (λ_{max} 450 nm, ε = 18,100 M^{-1} cm^{-1}).



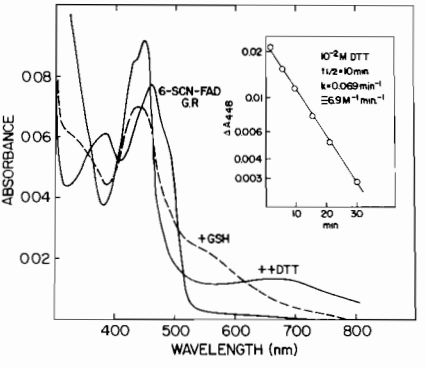


Figure 1. (left) Conversion of 6-SCN-FAD glutathione reductase to the 6-mercapto-FAD form by reaction with DTT. The spectrum of the 6-SCN-FAD enzyme is that with λ_{max} of 456 and 380 nm. Immediately after addition of 3 mM DTT the spectrum changed to that of curve 1. This was followed by the slow secondary change shown by curves 2-10. Conditions; 0.05 M phosphate + 0.3 mM EDTA, pH 7.0, 20°C.

Figure 2. (right) Effect of reduced glutathione followed by DTT on 6-SCN-FAD glutathione reductase. On addition of 2 mM GSH (K_d ~ 0.1 mM) the dashed-line spectrum was obtained and remained unchanged over a period of more than 1 hour. On the subsequent addition of 10 mM DTT, the 6-mercapto-FAD enzyme was formed with a $t_{½}$ of 10 min (see inset). Conditions, as in Fig. 1.

The rate of conversion of the 6-SCN-FAD enzyme to the 6-mercapto-FAD form is dependent on the concentration of DTT employed. The inset to Figure 1 shows a semi log plot of the absorbance increase at 448 nm; an average second order rate constant of 4.5 M^{-1} min^{-1} was obtained in several such experiments. These results indicate that the conversion is brought about by DTT itself, rather than by interaction of the enzyme active site dithiol with the flavin 6-SCN substituent. This conclusion was confirmed by the finding that reduced glutathione (GSH) produced the same EH_2 spectrum as found initally with DTT, but failed to bring about the secondary conversion, even on prolonged incubation. Several lines of evidence indicate that DTT gains success to the flavin 6-SCN substituent via the pyridine nucleotide pocket on the re-side (8) of the flavin. First, DTT added after saturating GSH brings about the conversion to 6-mercapto FAD, as shown in Figure 2. In fact the second order rate constant is slightly greater than that found in the absence of GSH, perhaps indicating some distortion of the active site to make the flavin re-face more accessible when GSH is bound (presumably as the mixed disulfide with Cys 58). Second, $NADP^+$ binds tightly to the EH_2 form produced by GSH, and blocks completely the subsequent conversion to the 6-mercapto-FAD form by DTT (results not shown).

The spectrum of the 6-mercapto-FAD enzyme shown in Figure 1 is that with the active site disulfide reduced. On removal of DTT and air reoxidation the long wavelength peak at 680 nm shifts to 720 nm with essentially the same extinction coefficient. This effect is readily reversed by DTT. The spectrum is that typical of the anionic species of 6-mercapto flavin (9) in which the negative charge is localized in the N(1)-C(2) locus of the flavin. When bound to GR the anionic species is highly stabilized, the spectrum being unchanged on dialysis of the 6-mercapto-FAD enzyme vs 0.1 M acetate, pH 5.0. Thus the pK of 5.9 with the free flavin (9) is lowered by at least 1.5 pH units on binding to the protein. This might be attributed to the stabilizing effect of a protein α-helix whose N-terminal end contributes a strong partial positive charge directed toward the N(1)-region of the flavin (8).

Crystal Structure of 6-mercapto-FAD Glutathione Reductase

Crystals of the 6-mercapto-FAD enzyme were grown by the hanging drop method using the same crystallization conditions as with native enzyme (1). The crystals, obtained within three weeks, diffracted to at least 2A° resolution. They are much better ordered than the crystals of apoprotein and appear to reach the quality of the native crystals. The space group and unit cell parameters are identical with those of the native enzyme. We collected 10,934 reflections up to 3A° resolution with an average internal R value of 8.3% using a 4-circle diffractometer (1).

For identification of the structural changes, the difference map between the electron density of the 6-mercapto-FAD enzyme and native enzyme was used. The structure factors of the model were taken from the 1.54A° crystal structure of glutathione reductase refined to an R-factor of 18.6% (10). The structure of the 6-mercapto-FAD enzyme was refined at 3A° resolution by a restrained least-squares procedure (11) to an R-factor of 13.7% with an overall root-mean-square deviation of 0.02A° for bond lengths and 2.1° for bond angles.

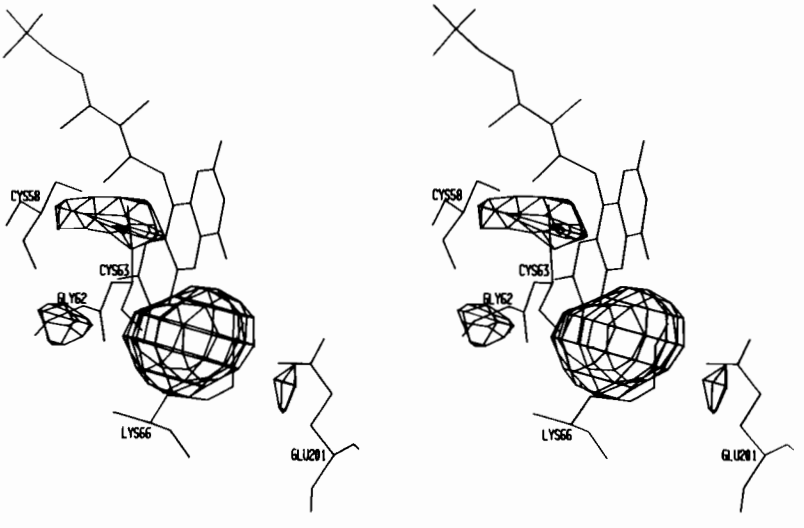


Figure 3. Positive difference-fourier map around the 6-mercapto group of 6-mercapto-FAD glutathione reductase, and native enzyme. The contours are drawn at 15% of the maximum of this map.

The highest peak in the difference fourier map (Fig. 3) was four times higher than the second highest peak, and corresponds unequivocally to the sulfur of 6-mercapto-FAD. The bond length between the S6- and C6-atoms is about 1.6A°, strongly supporting the double bond character of the flavin-sulfur linkage in the anionic 6-mercapto flavin (expected single bond length = 1.8A°, expected double bond length = 1.7A°).

The model of the native enzyme (10) shows free space within a radius of about 5A° around the C6-atom of the flavin. This space clearly facilitates the incorporation of 6-SCN-FAD and explains why there are only minor alterations of the enzyme structure and the FAD-binding position with 6-mercapto-FAD. The 6-mercapto group is surrounded directly by residues Gly 62, Cys 63, Lys 66 and Glu 201. The electron density map of Fig. 3 reveals that Lys 66 is shifted only a fraction of an Angström away from the flavin 6-S linkage. The carboxyl group of Glu 201, which forms a strong salt bridge to Lys 66, is moved about 0.2A° sideways, evading the sulfur of 6-mercapto-FAD. The carboxyl group of Gly 62, which has only little mobility in the native enzyme (10), is shifted about 0.2A° away from the sulfur. This effect, and the spatial proximity of Cys 63 to the 6S-atom of the FAD, induces a small movement of the cystine bridge Cys 58:Cys 63 in a direction parallel to the plane of the isoalloxazine moiety.

The above results confirm fully the conclusions reached from the study of the conversion of the 6-SCN-FAD enzyme to the 6-mercapto-FAD form by DTT. The 6-SCN residue can be readily accomodated by the flavin-binding pocket of the enzyme, and be available for attack by DTT from the nicotinamide side of the flavin. On the other hand, with a distance of about 6.3A°, the sulfur of the 6-SCN group would be too far away from the active site disulfide for the conversion to occur through reduction of the disulfide, and the 6-SCN group would not be accessible to DTT from the glutathione side of the flavin because of steric hindrance. These results are in sharp distinction to those obtained with mercuric reductase (5) and point to significant differences in the active sites of the two enzymes.

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