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GLUTATHIONE REDUCTASE SPECIES CONTAINING FAD ANALOGUES

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INTRODUCTION

Glutathione reductase (GR) (1,2,3) is a suitable enzyme for correlating spectroscopic properties and chemical reactivities of protein-bound flavin analogues with structural data. As reported here FAD, the prosthetic group of the enzyme, was replaced by analogues which carried modifications at the positions 8,6,4,2 and 1 of the isoalloxazine ring.

RESULTS AND DISCUSSION

Binding of 8-mercapto-FAD to apoglutathione reductase causes a shift of λ_{max} from 530 to 560 nm. Within 12h this intermediate changes to a final stable spectrum with absorption maxima at 575 and 455 nm (Fig.) The spectral changes probably reflect a very slow protein rearrangement subsequent to a primary binding step. 8-Mercapto-FAD is bound to apo·GR predominantly in the blue p-quinoid form which carries a negative charge in the pyrimidine subnucleus (4). This charge is probably neutralized by the positive pole of helix 338-354 (5,6). 8-Mercapto-FAD-GR possesses 40% of the enzyme activity of native FAD·GR. It reacts readily with thiol reagents such as methylmethane thiosulfonate (MMTS) (k_2= 1.4 x 10^5 $M^{-1}cm^{-1}$) to give a spectrum characteristic of an 8-S-"alkylated" flavin (7). The conversion is complete with a stoichiometric amount of MMTS so that side reactions with cystein residues of the enzyme in the oxidized form (E(1,8)) can be excluded.

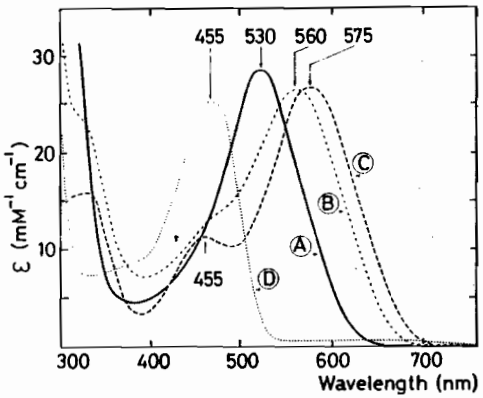

Fig.: 8-Mercapto-FAD in phosphate buffer, pH 6.9 (curve A) was titrated with apoglutathione reductase at 25°C. The spectrum was recorded immediately after titration (curve B) and 12h later (curve C). Curve D resulted from the reaction of 8-mercapto-FAD·GR (curve C) with one equivalent of methylmethane thiosulfonate.

The reaction is completely reversible by using 10 mM 2-mercaptoethanol. Reduction of 8-mercapto-FAD·GR with one equivalent NADPH leads to a slight hypsochromic shift of the long wavelength maximum to 568 nm and a decrease of the intensity of the 568 nm and 444 nm bands by 10%. No absorption increase at 340 nm is observed, so that this species is likely to represent 8-mercapto-FAD·EH_2. The spectrum does not show the characteristic "charge transfer" absorption of native EH_2 (1,8). Reduction of the flavin is achieved by a large excess of NADPH: the resulting species is 8-mercapto-$FADH_2$·EH_2 (=EH_4). Enzyme bound 8-Cl-FAD or 8-F-FAD reacted only poorly with sulfide to give 8-mercapto-FAD·GR. At most, 50% conversion was obtained by incubating 8-F-FAD·GR with 20 mM HS^- at pH 8.8 for 20h at 25°C.

The chemical reactivities at position 8 can probably be explained in structural terms. In native GR the xylene ring of FAD around the 7α-methyl group sticks in a very hydrophobic pocket. C(8) is in van-der-Waals contact with the oxygen atom of the peptide bond of Val61. The 8α-methyl group is at a distance of 4 Å to N_η of Arg291. These steric constraints may explain why, in the cases of 8-Cl-FAD·GR and 8-F-FAD·GR, a nucleophilic substitution by HS^- is hindered whereas the reaction of the 8-mercapto-FAD enzyme with MMTS is fast.

Addition of an equimolar amount of apo·GR to 6-OH-FAD does not alter the spectrum of the chromophore appreciably. A pH titration of the complex leads to spectral changes which however, cannot be described by a single ionisation process. The estimated pK for the changes at 330 and 680 nm is 7.2 ± 0.3. In contrast to the other GR species tested, 6-OH-FAD·GR has no detectable catalytic activity. This is not surprising in light of the three-dimensional structure of native FAD·GR. In native GR three amino acid residues, namely Gly62, Cys63 and Lys66, are in van-der-Waals contact to position C(6)(G.E.Schulz, personal communication). Introduction of an OH group at this position must disturb the protein structure, and/or the modified flavin is likely to be bound weakly. Poor binding is consistent with the observation that ultrafiltration of 6-OH-FAD·GR leads to the loss of the chromophore.

Reconstitution of apo·GR with 4-thio-FAD leads to a shift of the absorption maximum from 486 to 504 nm. This enzyme species shows 24% catalytic activity when compared with native FAD·GR. Reduction of the flavin in 4-thio-FAD·GR was achieved with a 1.9 fold molar excess of NADPH under anaerobic conditions. In this respect the 4-thio-FAD enzyme differs from all other GR species tested. The native enzyme, 8-Cl-FAD·GR and 1-deaza-FAD·GR for instance are reduced by NADPH to EH_2 species, which are characterized by (re)oxidized flavin and a dithiol group at the catalytic site (1,3). 4-Thio-FAD·GR does not react with methylmethane thiosulfonate (MMTS) and it reacts very

slowly with H_2O_2. Excess H_2O_2 leads to isosbestic formation of a new enzyme species with maxima at 452 and 364 nm which shows no catalytic activity. This species possibly contains FAD covalently bound to a side chain of the protein. Lys66 with its ε-NH_2 group at a distance of 3 Å to position O(4α) in the native enzyme, would be a good candidate; the long side chain of this residue lines the flavin around C(6)/N(5)/O(4α)(6).

When 2-thio-FAD binds to apoglutathione reductase, the absorption maxima shift from 486 to 504 nm and from 316 to 324 nm, respectively. 2-Thio-FAD·GR possesses 17% of the activity of native FAD·GR. The 2-thio-FAD enzyme was reacted with MMTS in order to test the accessibility of the region N(1)-C(2)-N(3) in the bound flavin. Even drastic conditions (10mM reagent, 24h incubation time, pH 9 at 25°C) did not lead to the expected spectral changes, which indicates that the pyrimidine subnucleus is unaccessible to solvent borne reagents. This is consistent with structural data. The peptide NH group of Thr339 forms an H-bond with N(1)/O(2α). In addition some other residues of the α-helix 338-354 are in close contact to N(1)/O(2α)/N(3) of the flavin. The strong hydrogen bond between N(3) and the carbonyl group of His467 is also important (6).

Upon incubation of 1-deaza-FAD with apo·GR, the long wavelength band of the chromophore is shifted from 535 to 560 nm and the intensity of the 365 nm band increases by 70%. The complex is stable at 2°C but tends to precipitate at room temperature, and also upon aerobic addition of NADPH at 2°C. Anaerobic addition of NADPH leads to a species resembling EH_2 of the native enzyme. Evidence for this is the disappearance of the absorption around 340 nm in the absence of flavin reduction. λ_{max} is shifted from 560 to 540 nm but the spectrum lacks the long wavelength absorption band of native EH_2. 1-Deaza-FAD·GR was found to have about 22% activity when compared with native FAD·GR. This means that an H-bond between protein and atom-1 of flavin is not essential for enzyme activity.

In conclusion, FAD-analogues are promising tools for mechanistic and drug designing studies on glutathione reductase. As to the latter point, inhibitors of GR are used clinically in the chemotherapy of malignancies and experimentally in malaria research. The structurally unstable 1-deaza-FAD·GR, for instance, is of interest because it resembles the GR species found in hereditary glutathione reductase deficiency of human erythrocytes (9). This condition, like favism, is believed to provide some protection against Plasmodium falciparum malaria.

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