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AN ESSENTIAL HISTIDINE RESIDUE IN THE CATALYTIC MECHANISM OF MAMMALIAN GLUTATHIONE REDUCTASE

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SUMMARY: Glutathione reductase from human erythrocytes was inactivated by ethoxyformic anhydride, and > 95 % activity was lost by modification of about 1-1.5 histidine residues per flavin (or subunit), as measured by the increased absorbance at 240 nm. Full reactivation was obtained with hydroxylamine. The rate of inactivation increased with pH and an apparent pK = 5.9 was obtained for the protolytic dissociation. The modified enzyme was inactive with NADPH and GSSG as substrates, but almost fully active in catalysis of a transhydrogenase reaction involving pyridine nucleotides. The visible absorption spectrum of oxidized or two-electron-reduced enzyme was not changed, but the flavin fluorescence of oxidized enzyme increased 2-fold after the modification. NADPH or $NADP^+$ did not protect the enzyme against inactivation. It is concluded that the modification affects a histidine involved in the second half-reaction of the catalysis, i.e. reduction of GSSG by the dithiol of reduced enzyme. Glutathione reductase from three additional mammalian sources was similarly inactivated, but enzyme from yeast was much less inactivated by the corresponding treatment with ethoxyformic anhydride.

INTRODUCTION

Glutathione reductase catalyzes the reduction of glutathione disulfide by NADPH as well as transhydrogenase reactions, such as that involving NADPH and thionicotinamide adenine dinucleotide phosphate (cf. [1] for a recent review). Two chemical constituents of the active center of the enzyme have been identified, FAD and a redox-active disulfide of cystine. Recent X-ray diffraction analyses [2,3] have shown that distinct binding sites exist for pyridine nucleotides and for disulfide substrates, and that the sites are located on opposite sides of a subunit of the protein and separated by the isoalloxazine ring of FAD. However, there is no additional chemical information on catalytically active groups at the active site, even if a base has been implied in the reaction mechanism [4]. The present communication gives evidence for the presence of histidine involved in

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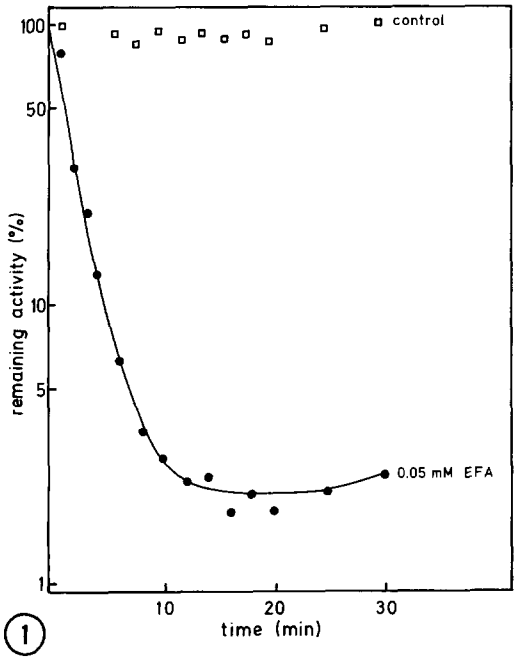
catalytic events in the disulfide-binding site of glutathione reductase.

MATERIALS AND METHODS

Glutathione reductase from human erythrocytes was purified essentially as described in [5]. The enzyme was homogeneous as judged from the specific activity (about 240 units/mg), polyacrylamide gel electrophoresis and the flavin spectrum. The conditions for the assay of glutathione reductase and transhydrogenase activities have been described elsewhere [6]. All the chemicals used were commercial products of high purity. Stock solutions of ethoxyformic anhydride were previously prepared in 99.5 % ethanol and the concentration was determined as described in [7]. Ethoxyformylation of the enzyme was normally carried out at 30°C in 50 mM sodium acetate buffer (pH 6.0) containing 1 mM EDTA. The enzyme had been dialyzed previously against the same buffer. Spectral measurements were made in a Beckman model 25 or in a Cary model 17 spectrophotometer.

RESULTS

Treatment of glutathione reductase with 50 μM ethoxyformic anhydride gave > 97 % inactivation within 10 min at pH 6.0 (Fig. 1). Addition of $NADP^+$ or NADPH to the incubation mixture gave no protection of the enzyme against the inactivator. The effect of added GSSG could not be properly evaluated because GSSG reacted directly with ethoxyformic anhydride and a possible protection by binding to the enzyme could not be discerned. The pH-dependence of the inactivation was studied and the apparent pseudo-first-order rate constants are plotted versus pH in Fig. 2. Apparently a protolytic dissociation with a pK_a of about 5.9 affects the inactivation reaction. The degree of inactivation was established by measuring the reduction of GSSG by NADPH. When the transhydrogenase activity was assayed, only about 15 % inactivation was found under the conditions giving > 95 % inactivation of the reaction involving GSSG. The inactivated enzyme was fully reactivated by addition of 0.6 M hydroxylamine. The pH dependence and the reactivation experiments indicated that histidine was modified [7], and this interpretation was further supported by the increase in optical absorbance at 240 nm [7]. From the absorbance increase it could be calculated that 1-1.5 histidine residues per flavin (or subunit) had been modified when the activity was inhibited > 95 %. The rate of histidine modification, as measured at 240 nm, was the same as the rate of inactiva-



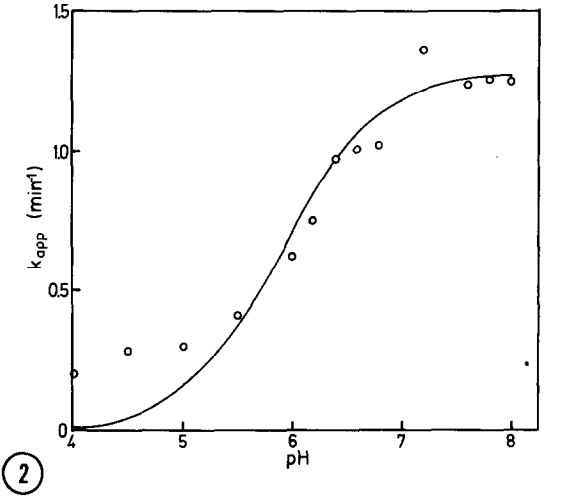


Fig. 1. Inactivation of glutathione reductase from human erythrocytes by ethoxyformic anhydride (EFA). The enzyme was preincubated at 30°C with (●) or without (□) the reagent (50 µM) at pH 6.0 and aliquots of the reaction mixture were analyzed with GSSG and NADPH for remaining catalytic activity.

Fig. 2. Dependence on pH of the inactivation rate of glutathione reductase from human erythrocytes. The enzyme was incubated with 50 µM ethoxyformic anhydride in 50 mM citrate-phosphate buffers of different pH values. Pseudo-first-order rate constants for the inactivation were obtained from the initial parts of the time-progress curves. An equation describing the dependence of the rate constant on a single protolytic dissociation was fitted by nonlinear regression to the experimental data. From this analysis was derived a pK value of 5.9 governing the rate of the inactivation reaction.

tion of the enzyme within the limitations of the experimental error, indicating that the histidine modified is essential for activity. The visible absorption spectrum of modified enzyme was indistinguishable from native oxidized enzyme, and the absorbance in the wavelength region around 530 nm, typical of the two-electron-reduced enzyme [4], was readily obtained by reduction of modified enzyme with NADPH. Thus, an effect of ethoxyformic anhydride on the isoalloxazine ring of FAD can be excluded. However, the fluorescence of the enzyme-bound FAD, which is highly quenched in the in-

active enzyme, increased 2-fold (measured at the emission maximum at 516 nm; excitation at 450 nm). This observation is consistent with the assumption that the modified group is at the active site close to FAD.

DISCUSSION

The findings reported above lend strong support to the identification of a histidine residue, which is essential for the catalytic activity of glutathione reductase. The fact that no protection against ethoxyformic anhydride was obtained with $NADP^+$ and NADPH as well as the limited effect on the transhydrogenase activity of glutathione reductase indicate that the histidine residue modified is at or near the GSSG binding site. This conclusion is supported by the requirement of a base in the reaction between the redox-active disulfide of the enzyme and GSSG [4,8]. In the recent X-ray diffraction analysis a histidine residue has indeed been tentatively identified near the isoalloxazine ring and on the same side as the redox-active disulfide and the GSSG-binding site [3]. We assume that this amino acid residue (in position 450 the sequence of the human enzyme) is the group modified by ethoxyformic anhydride. The proposed function of the histidine is in the second half-reaction of the catalysis in which two-electron-reduced glutathione reductase reacts with GSSG to yield 2 GSH and reoxidized enzyme. The first half-reaction, which involves the reduction of the enzyme by NADPH, is apparently not directly dependent on the residue modified, since the enzyme is reducible and the transhydrogenase activity essentially unaffected by the chemical modification. We have earlier proposed that only the two electrons and not the two protons required for reduction of GSSG to GSH are transferred from the pyridine-nucleotide-binding site to the GSSG-binding site of a subunit of the enzyme [1]. The two protons are assumed to be taken up one at a time via the GSSG binding site on the other side of the subunit.

Fig. 3 shows the chemical groups assumed to participate in the catalysis. The sulfur interacting with the isoalloxazine ring of FAD is Cys-46

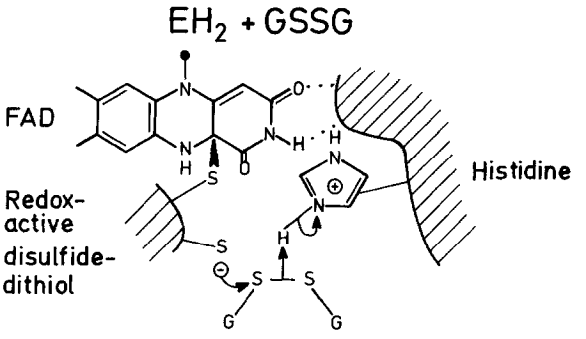


Fig. 3. Scheme of the active center of one subunit of mammalian glutathione reductase. The GSSG-binding site [2,3] is in front of the isoalloxazine ring of FAD. The sulfur atom interacting with the isoalloxazine ring originates from Cys-46 and the negatively charged sulfur from Cys-41 of the redox-active disulfide of human glutathione reductase [3,5]. The imidazole ring belongs to the histidine modified by ethoxyformic anhydride (possibly the group, which has tentatively been identified as histidine in position 450 in the amino acid sequence of the second subunit [3]). It is assumed that in catalysis the histidine, on the one hand, increases the nucleophilicity of Cys-41 by promoting the dissociation of a proton, and, on the other hand, serves as an acid which protonates the sulfur of the leaving half of GSSG (yielding GSH).

and the second sulfur atom of the protein is Cys-41 both belonging to the FAD-binding domain of one subunit [3]. The imidazole ring of a histidine is the group modified by ethoxyformic anhydride. The histidine is probably residue 450 of the second subunit [3]. The results of the present investigation show that the modification of glutathione reductase with ethoxyformic anhydride has only a limited effect on the reactions involving pyridine nucleotides (reduction of the enzyme with NADPH and transhydrogenase activity). Thus, the histidine probably is not directly involved in the generation of two-electron-reduced enzyme by means of NADPH (the first half-reaction of the catalysis). Since the GSSG-reduction is drastically affected by the modification (Fig. 1), it is evident that the role of the histidine must be played in the reoxidation of enzyme by means of GSSG (the second half-reaction of the catalysis). It is proposed that the catalysis involves transfer of reducing equivalents from NADPH in the pyridine-nucleotide-

binding site of a subunit, via the isoalloxazine ring, to the disulfide group formed by Cys-41 and Cys-46 near the GSSG-binding site on the other side of the isoalloxazine ring of the same subunit. In this first half-reaction histidine has no direct role, but when a negative charge develops on the sulfur of Cys-41 upon reduction of the disulfide bond, the histidine may assist in the uptake of a proton which will neutralize the negative charge. The proton may be shared between Cys-41 and the histidine ("His-450" [3]); in Fig. 3 it is placed on the imidazole ring to show how the imidazolium ion could serve as an acid, which protonates one of the sulfur atoms of GSSG concurrent with a nucleophilic attack by the thiolate of Cys-41 on the other sulfur atom of GSSG. If the imidazole group is modified by ethoxyformic anhydride, it can neither promote the dissociation of a proton from the sulfhydryl group of Cys-41 in reduced enzyme, to form the nucleophilid thiolate, nor act as an acid in the formation of GSH from GSSG. In the last phase of the normal catalysis it is assumed that the histidine takes up a new proton from the medium in the GSSG-binding site and protonates the second glutathione moiety in an acid-catalyzed cleavage of the intermediary mixed disulfide of Cys-41 and glutathione. Neither can this reaction be catalyzed by the modified enzyme.

An interacting cysteine-histidine pair has been found in the active center of papain [9,10] and been found to have a pK value of histidine which is 4.3 when the cysteine is protonated and 8.5 when the cysteine is deprotonated [11]. Likewise, the pK of the thiol group increases from 3.3 to 7.6 on deprotonation of the imidazole group [11]. Such a thiolate-imidazolium ion pair has previously been discussed for lipoamide dehydrogenase and glutathione reductase [8]. It is expected that the modification of histidine of glutathione reductase with ethoxyformic anhydride prevents protonation of the imidazole ring and accordingly makes ionization of Cys-41 in reduced enzyme more difficult and the sulfhydryl group less reactive. The apparent pK value of 5.9 for the ionization affecting the

modification of glutathione reductase (Fig. 2) must be referred to the oxidized (disulfide) form of the enzyme, because the inactivation reaction is carried out with oxidized enzyme. The pK value for a possible cysteine-histidine pair in the reduced enzyme cannot be expressed under the conditions used.

The results reported in the present paper refer primarily to glutathione reductase from human erythrocytes, but very similar results were obtained with the enzyme from other mammalian sources such as porcine erythrocytes, calf liver, and rat liver. However, the enzyme from yeast was only slightly (less than 10-15 % inhibition) inhibited by ethoxyformic anhydride. This finding indicates a difference in the reactive groups of the enzyme from yeast and mammalian sources.

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