Electron Paramagnetic Resonance Identification of a Highly Reactive Thiol Group in the Proximity of the Catalytic Site of Human Placenta Glutathione Transferase*

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The reaction of the glutathione transferase from human placenta with a maleimide spin label derivative has been followed by EPR. Incubation of the enzyme at pH 7.0 with 50-fold molar excess of the spin label reagent gives rise to an immobilized nitroxyxl EPR spectrum indicative of two reacting thiol groups per dimer of enzyme as evaluated by double integration of the EPR spectrum; the activity is lost in parallel. The same type of spectrum can be obtained simply by adding 2 eq of the spin label reagent to the enzyme. The binding is completed after less than 1 min at pH 8.0; it requires 2 min at pH 7.0 and more than 10 min at pH 6.0. These data indicate that the maleimide derivative reacts, in each subunit, with a thiol group which plays a crucial role for the maintenance of the catalytic activity and is characterized by a low pH. Inactivation of the enzyme at pH 7.0 in the presence of 2 eq of spin label reagent per mol of enzyme requires 15 min, suggesting the occurrence of a structural rearrangement after the binding of the thiol blocking agent. The same binding in the presence of S-methyl glutathione or protoporphyrin IX shows a decreased reaction rate with respect to the reaction in the absence of inhibitors, indicating that the thiols are in proximity of both the glutathione and the porphyrin binding sites. For this latter case, this is unambiguously demonstrated by the titration of spin-labeled enzyme with hemin, which produces a decrease of the EPR signal amplitude from which an interspin distance of about 10 Å can be evaluated.

EXPERIMENTAL PROCEDURES

Sl-maleimide and 4-amino-TEMPO were purchased from Aldrich, protoporphyrin IX and hemin from Fluka, S-methylglutathione and DTNB from Sigma. All other chemicals were of reagent grade.

The GSH transferase was purified from human placentas by a slight modification of the procedure used for the human heart enzyme (13). The tissue, homogenized with 4 volumes of 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol, was subjected to one step of purification on a GSH affinity column (14) followed by chromatofocusing on an LKB fast protein liquid chromatography unit equipped with a Mono P HR 5/20 Pharmacia LKB Biotechnology Inc. column, as described elsewhere (13). The purity of the enzyme was tested by polyacrylamide gel electrophoresis in sodium dodecyl sulfate according to the method of Weber and Osborn (15). The GSH transferase activity was measured spectrophotometrically (340 nm) at 25°C in 1 ml of 0.1 M potassium phosphate buffer, pH 6.5, containing 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (16); the resulting specific activity was 80 units/mg (1 enzymatic unit catalyzes the conjugation of 1 μmol of substrate per min). The protein concentration was determined by the method of Bradford (17). Protein sulfhydryl groups were determined by the method of Ellman (18). Spin-labeled GSH transferase was prepared by incubation of 130 μM enzyme in 10 mM potassium phosphate buffer pH 7.0, with 50 excesses of sl-maleimide for 5 min at 37°C. The unreacted spin-labeling reagent was removed by dialyzing the sample against 10 mM potassium phosphate buffer, pH 7.0. The extent of spin label reacted with GSH transferase was estimated by double integration of the EPR signal before and after denaturation of the protein upon addition of concentrated NaOH. The resulting numerical value was compared to that obtained from a standard solution of 4-amino-TEMPO. The

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1 The abbreviations used are: sl-maleimide, N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).
kinetics of the sl-maleimide binding were determined by adding sl-maleimide at 1:1 subunit/label ratio to GSH transferase samples buffered at a known pH and recording an EPR spectrum every 50 s. The samples at pH 6.0 and 7.0 were prepared by dialyzing aliquots of 100 μM GSH transferase against 10 mM potassium phosphate buffer, pH 6.0 or 7.0, respectively. After dialysis, the GSH transferase was dialyzed to the appropriate concentration for the EPR measurement, usually 40 μM. The sample at pH 8.0 was prepared by addition, just before the EPR experiments, of diluted KOH to a GSH transferase solution at pH 7.0, to avoid as much as possible the oxidation of the protein thiol groups.

The kinetics of binding in presence of S-methylglutathione or protoporphyrin IX were determined after adding 2 eq of sl-maleimide to a GSH transferase solution buffered at pH 7.0 and incubated for 5 min at 25 °C with the appropriate concentration of the ligand. The kinetics of the enzyme inactivation was followed by taking, at different times, aliquots of 5 μl from a 10 mM potassium phosphate buffer pH 7.0 solution containing 1.24 μM GSH transferase and 2 eq of sl-maleimide; the GSH transferase activity was measured as described above. Titrations of the spin-labeled enzyme with hemin or protoporphyrin IX were made at pH 7.0 by adding aliquots of the porphyrins to a solution of 65 μM labeled GSH transferase.

EPR spectra were run on an ESP 300 Bruker spectrometer operating at 9 GHz. Temperature was maintained at 25 °C with a Bruker temperature control system.

RESULTS

The EPR spectrum of the GSH transferase incubated for 5 min at 37 °C with a 50-fold molar excess of sl-maleimide and then dialyzed against 10 mM potassium phosphate buffer, pH 7.0, to remove the unreacted compound, is shown in Fig. 1A. The spectrum is mainly characterized by a strongly immobilized nitroxide species (ΔA1/2 = 64 G) and by a small component accounting for less than 5% of the total signal and characteristic of free nitroxide (Fig. 1B) not completely removed by dialysis. Double integration of the EPR signal indicates 2 mol of nitroxide label bound per mol of enzyme.

An EPR spectrum with identical line shape could be obtained simply by adding to the enzyme 2 eq of maleimide spin label. In this condition, the kinetics of the binding can be followed easily, taking advantage of the fact that the free and bound spin labels have clear and distinguishable EPR signals. The kinetics of binding of 2 eq of sl-maleimide at pH 7.0 and 8.0 indicated a very fast reaction (t1/2 = 50 s at pH 7.0 and t1/2 ≤ 20 s at pH 8.0). The time-dependent inhibition of GSH transferase activity by 2 eq of sl-maleimide at pH 7.0, shown in Fig. 2, indicates two distinct phases: the first one ends in less than 2 min with 50% inhibition of activity, while the second one is completed in about 15 min with a final enzyme inhibition of 80%. Titration of the SH groups of the protein with DTNB, before incubation with sl-maleimide, revealed the presence of four reactive sulphydryls. On the other hand, after the first 2 min of reaction with sl-maleimide, only two SH groups react with DTNB. They remain reactive after 20 min or more. A plot of the disappearance of the free label EPR signal against time is also reported in the inset of Fig. 2. The kinetics of binding of sl-maleimide has also been measured at pH 6.0. In this case, the rate of disappearance of the free label is much slower, which allowed us to better understand the mechanism of binding. The kinetics of binding at pH 6.0 at 1:1 subunit/label ratio is reported in Fig. 3.

To gain information about the structural location of the 2 cysteine residues, the kinetics of the binding of the sl-maleimide were also followed at pH 7.0 in the presence of two compounds known to inhibit the activity of the GSH transferase (1), namely S-methylglutathione and protoporphyrin IX. The kinetics, reported in Fig. 4, indicate in both cases a decrease of the rate of reaction of the sl-maleimide. As a final experiment, a titration with either the paramagnetic hemin or the diamagnetic protoporphyrin IX on the sl-maleimide-
reacted enzyme was carried out. Protoporphyrin IX was found not to have any effect, either on the shape or on the intensity of the nitroxide EPR signal (results not shown), while hemin produced a decrease of the EPR intensity without any perturbation of the line shape (Fig. 5). A plot of the intensity of the EPR signal, evaluated by double integration, against the ligand concentration is reported in Fig. 6. The EPR signal of iron from the hemin was observed at liquid nitrogen temperature during the same titration. The signal, due to a high spin iron(III) with axial symmetry, is not perturbed by the presence of the protein suggesting that the heme-protein interaction does not happen through the metal but likely through the porphyrin ring.

**DISCUSSION**

The EPR spectrum of Fig. 1A is characterized, apart from a free label component accounting for less than 5% of the total signal, by a single bound species (2A_e = 64 G) which accounts for 2 mol of sl-maleimide reacted per mol of enzyme. In parallel, two sulphhydryl groups disappear as determined by titration with DTNB. The EPR signal of the bound spin-label is homogeneous and has a line shape completely identical with that displayed by enzyme which was reacted with 1 eq of spin-label reagent (spectrum not shown). This indicates that the reaction occurs with two identical thiol groups (i.e. one for each monomer) that confer the same physicochemical constraints on the label. The two thiols are very reactive, in fact, at pH 8.0, 2 eq of sl-maleimide can bind to the protein in less than 50 s. The reaction is also very fast at pH 7.0 (Fig. 2, inset), while the kinetics of inactivation of the enzyme displays a different time course (Fig. 2). In fact, two distinct phases can be detected; the first one with an inhibition of 50% ends in less than 2 min, a time that corresponds to that required for binding 2 eq of sl-maleimide at the same pH as detected by EPR. The second phase is much slower: it ends up in 15 min and reduces further the residual enzymatic activity. A likely explanation is that after the fast binding of sl-maleimide to the thiol groups, a slow rearrangement of the protein takes place to reach an almost inactive conformation. In line with this is the observation that after 2 min only two sulphhydryl groups are titrable with DTNB. The drastic inactivation (50%) observed after 2 min (Fig. 2), the time required for the binding of sl-maleimide, as detected by EPR, suggests a close proximity between the thiol group and the active site of the enzyme. This is in agreement with the experimental finding that the GSH transferase activity is strongly dependent on the integrity of the SH groups (8-11) and with the recent result that alklylation of this enzyme with a fluorescent maleimide probe gives rise to enzyme inactivation and selective modification of the Cys-47 residue (19). The binding reaction between the sulphhydryl groups and the sl-maleimide has been more thoroughly studied at pH 6.0 by taking advantage of the slower rate displayed by the reaction at this pH. The reaction kinetics are second order, with a calculated rate constant evaluated from the slope of the line of Fig. 3 of 110 ± 1 M⁻¹ s⁻¹ which is in agreement with the value obtained by plotting the pseudo-first order constants against enzyme concentration (data not shown).

Information about the location of the reacting thiol group comes from the kinetics of binding of the sl-maleimide in the presence of S-methylglutathione (Fig. 4). The reaction is slowed down with respect to that shown by the native enzyme, suggesting that this group is located near the GSH binding site (G site) (6), or that the binding of S-methylglutathione produces a conformational change able to screen the accessibility of such a group. A similar behavior is also displayed by the kinetics of disappearance of the free sl-maleimide in the presence of an excess of protoporphyrin IX (Fig. 4). The rate of binding decreases with respect to that obtained in the absence of protoporphyrin, again suggesting a close proximity between the thiol group and the heme binding site. The evidence of a close proximity between the porphyrin binding site and the reacting SH group also comes from the experiments reported in Fig. 6, where a plot of the EPR intensity of the sl-maleimide-reacted enzyme is reported as a function

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**Fig. 4. Pattern of reaction between sl-maleimide and GSH transferase in the presence of inhibitors.** sl-maleimide, at 1:1 subunit/label ratio, was added to a solution of 10 mM potassium phosphate buffer, pH 7.0, containing 40 μM GSH transferase preincubated with 2.5 mM S-methylglutathione (●) or 300 μM protoporphyrin IX (▲). The plot reports the fractional free label intensity as a function of time. For comparison, the trend of the same reaction in the absence of inhibitors is also included (▲).

**Fig. 5. EPR spectra of spin-labeled GSH transferase as a function of hemin concentration.** 65 μM spin-labeled GSH transferase in 10 mM potassium phosphate buffer, pH 7.0 (a), was titrated with 20 (b), 40 (c) and 80 (d) μM hemin. The first integral of the spectra is shown to emphasize the difference in intensity. EPR conditions were as in Fig. 1.

**Fig. 6. Intensity of the EPR spectra of 65 μM spin-labeled GSH transferase as a function of porphyrin concentration.** The EPR intensity of the spectra presented in Fig. 5, evaluated by double integration, is reported as a function of hemin concentration (●). The EPR intensities for the corresponding titration with the diamagnetic protoporphyrin IX are also shown for comparison (▲).
of the paramagnetic hemin or the diamagnetic protoporphyrin IX concentrations. The reduction of the label signal intensity is evident only for the titration with hemin and can be interpreted as due to a dipole-dipole interaction occurring between the maleimide nitroxy1 group and the iron. The theory to describe the line shape of an EPR signal which is influenced by dipolar coupling to a second spin in a rigid arrangement has been developed by Leigh (20) and has been applied to several systems. According to this theory, the distance between the spin label moiety and the Fe$^{3+}$ atom of the heme bound to the enzyme is estimated from the magnitude of the interaction coefficient $C$, which may be obtained from the measured fractional diminution in the amplitude of the EPR signal and the measured line width of the signal in the absence of paramagnetic interaction. The interaction coefficient $C$ is related to interspin distance by the equation:

$$C = g^2 \mu^2 r^3 / \mu^2$$

where $g$ is the electronic $g$ factor of the observed spin, $\mu$ is the magnetic moment of the paramagnetic ion, $r$ is the distance between Fe$^{3+}$ and the spin label. Because the iron from which a label-iron distance of $10^{-10}$-10$^{-11}$ (21) was taken for the iron from which a label-iron distance of 10 Å < $r$ < 14 Å was obtained. The highly reactive thiol group important for activity must then be located in between or across the hydrophobic ligand and the substrate binding sites in agreement with that found in rat liver ligandin (8) and in horse GSH transferase (11). Moreover, this group is characterized by a low pK, and its proximity to the active site, this could be determined by the same environment that induces a lowering of the pK of the enzyme-bound GSH in the active site (22). In addition, the kinetics of the enzyme inactivation observed after the binding of al-maleimide indicates that the cysteine integrity plays a crucial role for the maintenance of a catalytically active enzyme conformation.

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