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SUBSTRATE-INDUCED REDOX CHANGE OF SELENIUM IN GLUTATHIONE PEROXIDASE STUDIED BY X-RAY PHOTOELECTRON SPECTROSCOPY

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Summary

Glutathione peroxidase showed an X-ray photoelectron spectroscopy signal of the Se 3d (3/2, 5/2) electrons at 54.4 eV. After the addition of the acceptor substrate H_2O_2, a marked shift of this signal to a value of 58.0 eV was observed. Upon subsequent treatment with the donor substrate glutathione, this chemical shift was reversed and the original signal was obtained. These data demonstrate that the enzyme-bound selenium moiety participates in the catalytic process. From the chemical shift obtained it is concluded that the enzyme shuttles between a selenol or selenol derivative in its reduced form and a seleninyl or selenonyl compound in its oxidized form.

Since selenium was discovered as an essential and biochemically active trace element by Schwartz and Foltz [1] 17 years ago, it has been found to be present in a protein isolated from lamb muscles [2], in formate dehydrogenase (EC 1.2.1.2) from *Clostridium thermoaceticum* [3] and other microorganisms [4], in the clostridial glycine reductase system [5], and glutathione peroxidase (glutathione: hydrogen-peroxide oxidoreductase EC 1.11.1.9) [6]. Preliminary studies indicate that the low molecular weight protein of glycine reductase contains 1 g atom of selenium per mol [5]. A stoichiometry of 4 g atoms of selenium per mol of GSH-peroxidase isolated from bovine blood was established by Flohé et al. [7] from this laboratory. This result has been confirmed for the ovine blood enzyme by Hoekstra et al. [8] and for the rat liver enzyme by Nakamura et al. [9].

The redox-sensitive character of selenium suggests that it may represent

Abbreviation: XPS, X-ray photoelectron spectroscopy.

an integral part of the catalytically active site of this selenoenzyme. At present, however, our knowledge on the chemical nature of the selenium-containing moiety of the enzyme is rather limited. A change in the oxidation state of the enzyme-bound selenium was merely postulated on account of kinetic studies [9]. Recently, X-ray photoelectron spectroscopy (XPS) proved to be a potent tool in identifying different oxidation states of organo-selenium compounds [10]. Since the chemical shift of the binding energy of the core electrons was expected to be rather distinct in the reduced and oxidized enzyme, we used this technique in order to elucidate the nature of the enzyme-bound selenium.

Glutathione peroxidase was prepared as described in ref. 12. A specific activity of 465 units/mg was obtained. 3.5 mg of desalted protein were applied to the adhesive side of a cellotape (Tesafilm). The oxidation of the enzyme was performed by spraying 50 μmol H_2O_2 aerosol onto the frozen enzyme. Reduction was achieved correspondingly by spraying 0.5 μmol of neutralized GSH onto the sample after H_2O_2 had been removed at a pressure of about 5 μTorr. The reference selenium compounds were applied either as a freeze-dried or as a finely ground powder. Since the exciting electron beam in this type of XPS measurement generates photoelectrons of the element under investigation only in a protein surface layer of a few Ångstroms [13], this substrate spray method seemed to be most convenient. It suffers from the disadvantage that no precise concentrations of substrates and no reaction time can be given.

XPS spectra were recorded at about $-100\,^{\circ}C$ and 1 μTorr on a Varian V-IEE 15 spectrometer equipped with an online Varian 620 L, 8 K computer. The energy of the exciting X-rays was 1253.6 eV (Mg $K\alpha_{1,2}$). The C 1s-line at 284.0 eV of the aliphatic carbon atoms of the protein served as internal standard. Instrumental conditions: X-ray source power of the magnesium anode was at 11 kV, 100 mA. Work function: 6.0 eV; analyzer energy: 100 eV; sweep width: 30 eV; sweep time: 20 s; number of scans: 100; number of channels: 200; sweep mode: sequential scans.

Glutathione (reduced) was purchased from Zellstoffwerk Waldhof-Aschaffenburg. Hydrogen peroxide, cadmium selenide suprapure, sodium selenite p.a. and selenium dioxide subl. were obtained from Merck A.G. Darmstadt, seleno-DL-methionine, seleno-DL-cystine, A grade, were products from Calbiochem, San Diego. Ribonuclease was obtained from Serva, Heidelberg.

The following reference model compounds which contain selenium in different binding states were synthesized Cys-Se-S-Cys by a disulfide exchange reaction according to [14]; the selenotrisulfide derivative of ribonuclease was obtained by a sulhydryl-selenotrisulfide-exchange reaction according to [15].

The X-ray photoelectron spectra of glutathione peroxidase are presented in Fig. 1a. Apart from an intensive Na 2s electron signal at 62.2 eV and an Mg 2p electron signal at 50.8 eV, spectrum (a) shows a relatively weak signal at 54.4 ± 0.5 eV. Since a peak of this intensity in this region of the spectrum

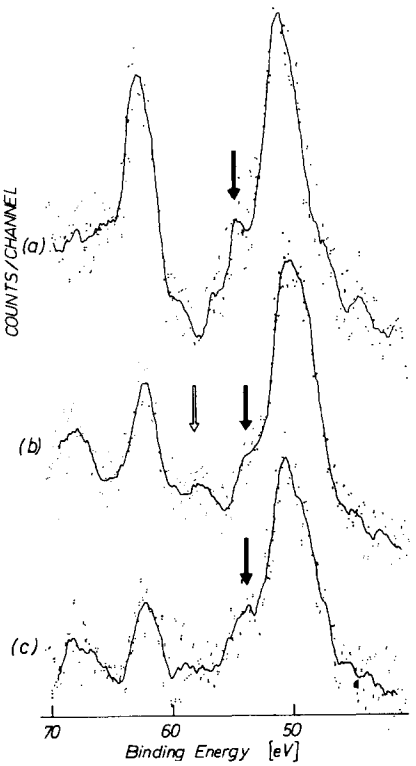

Fig.1. X-ray photoelectron spectra of glutathione peroxidase. 3.5 mg of pure enzyme were applied as a lyophilized powder. Arrows indicate the unresolved doublet Se 3d (3/2, 5/2) peaks lying between the Na 2s at 62.2 eV and the Mg 2p energy levels at 50.8 eV. (a) Spectrum of the freshly prepared enzyme. (b) Spectrum of the partly oxidized enzyme after treatment with 50 μmol of the acceptor substrate H_2O_2. (c) Spectrum of the reduced enzyme after oxidation by H_2O_2 and subsequent reduction by GSH. The binding energy of the Se 3d electrons shifted reversibly from 54.4 ± 0.5 eV in the reduced state to 58.0 ± 0.5 eV in the oxidized state and back to 54.1 ± 0.5 eV after reduction as indicated by the arrows. Spectra were smoothed over 15 points by a least squares smoothing function.

would have been expected to represent the binding energies of the Se 3d 3/2, 5/2 electrons, the spectra of various selenium-containing compounds were recorded. The binding energy levels obtained for the respective compounds were as follows (listed after increasing oxidation state): CdSe 53.6 eV; elemental selenium 54.8 eV; selenomethionine 55.1 eV; selenocystine 55.2 eV; the mixed thioselenide between selenocystine and cysteine 55.3 eV; the selenotrisulfide of ribonuclease 56.0 eV; sodium selenite 58.3 eV; selenium dioxide 58.7 eV. Comparison of these values with the spectrum (a) indicates that indeed the signal obtained at 54.4 eV for glutathione peroxidase corresponds to an enzyme-bound selenium moiety.

Consequently, we treated the sample stepwise with the two natural substrates of the enzyme and recorded the XPS-spectra. The Se 3d signal shifted from 54.4 ± 0.5 eV to 58.0 ± 0.5 eV after addition of H_2O_2 (Fig. 1b). This shift was reversed to 54.1 ± 0.5 eV by addition of the second substrate GSH (Fig. 1c).

The Se 3d levels seemed to be best suited for measuring the binding energies of selenium by XPS, because this peak shows the highest intensity for this element and does not overlap with signals of other bioelements. A possible interference of the Fe 3p core electrons can be ruled out since iron is absent in GSH-peroxidase [7]. Furthermore, in this region of the spectrum, a chemical shift of this magnitude following oxidation is highly unlikely for any other element [16], whereas similar shifts have been reported for organoselenium compounds [11]. The peak height obtained shows that as little as 14 μg of selenium bound in 3.5 mg of a large protein molecule can be detected by the XPS method, which has been successfully applied to other metalloproteins [17—19]. In addition, the Na 2s signal at 62.2 eV and the Mg 2p signal at 50.8 eV show the presence of large amounts of protein-bound sodium and magnesium.

The experimental results demonstrate that the element selenium in glutathione peroxidase undergoes a substrate-induced redox change. From this observation it may be concluded that selenium participates as the redox-sensitive component of the active site in the catalytic cycle of the enzymatic reaction. The obtained data suggest that the reduced enzyme in the absence of hydroperoxide does not contain a selenium-oxygen bond. This indicates that the selenium in this enzyme species may exist in an oxidation state corresponding to a selenol or a selenol derivative. From the chemical shift obtained following oxidation by H_2O_2 it may be concluded that in the oxidized enzyme an oxygen-containing selenium compound is formed. Whether a seleninyl- or a selenonyl-compound represents the oxidized enzyme form of the undisturbed enzymatic reaction cannot be decided. Reduction of this species by GSH leads to the original oxidation state. These results are consistent with the kinetic data obtained for this enzyme [10] as well as with a model presented by Günzler [20] who assumed the enzyme to shuttle between a selenol- and a thioseleninate state.

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