Biochemical characterization of selenium-containing catalytic antibody as a cytosolic glutathione peroxidase mimic

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A selenium-containing catalytic antibody (Se-4A4), prepared by converting reactive serine residues of a monoclonal antibody (4A4) raised against a GSH derivative into selenocysteines, acts as a mimic of cytosolic glutathione peroxidase (cGPX). To clarify the mechanism of action of this catalytic antibody, detailed studies on kinetic behaviour and biological activity were carried out. A rate of acceleration ($k_{\text{cat}}/K_m/k_{\text{uncat}}$) 10^7 -fold that of the uncatalytic reaction is observed. Under similar conditions, the turnover number (k_{cat}) of Se-4A4 is 42% of that of the natural

rabbit liver cGPX. The Se-4A4 reaction involves a Ping Pong mechanism, which is the same as that of the natural cGPX. The selenocysteine residue is located in the binding site of the antibody and is shown to be crucial for this activity. Of the thiol compounds tested, only GSH is able to serve as substrate for Se-4A4. It was demonstrated, using the free-radical-damage system (hypoxanthine/xanthine oxidase) of cardiac mitochondria, that Se-4A4 can protect mitochondria from free-radical damage at least 10^4 -fold more effectively than the natural cGPX.

INTRODUCTION

The selenium-containing peroxidases so far discovered form a family comprising four types of distinct but related gene products [1]. They catalyze the reduction of a variety of hydroperoxides (ROOH or H_2O_2) using GSH, thereby protecting mammalian cells against oxidative damage [2,3]. The best documented example is the cytosolic glutathione peroxidase (cGPX), which specifically uses GSH as the reducing substrate [4]. cGPX, first found in bovine erythrocytes by Mills [5], contains one selenocysteine (Sec) residue in each of the four identical subunits, which is essential for enzyme activity [6]. The structure and function of this enzyme have been described [4,7]. Each active site of cGPX, containing a Sec residue and the substrate GSH-binding site, is found in a flat depression on the molecular surface at 0.2 nm resolution. A series of GPX mimics (ebselen [8], glutathione [9], etc.) have been synthesized. However, the catalytic efficiencies of these mimics were found to be $10^{-3} \sim 10^{-4}$ times lower than that of the natural enzyme. Selenosubtilisin, reported by Hilvert and colleagues [10–12], exhibits GPX-like properties, but, unlike cGPX, it has no GSH-specific binding site in the active site. Therefore the selenosubtilisin catalysis of hydroperoxide reduction using an aryl thiol instead of GSH was studied.

Technology involving the generation of catalytic antibodies offers a new method of obtaining cGPX mimics. Because antibodies that selectively bind almost any molecule of interest can be generated, this new catalytic antibody technology offers the potential to make highly selective catalysts for applications in biology, chemistry and medicine [13,14]. We have previously described a selenium-containing catalytic antibody, Se-4A4, that mimics the catalytic behaviour of cGPX; it has a Sec residue incorporated into the binding site of antibody 4A4 raised against a GSH derivative [15]. The present report is a more detailed study of its biochemical characterization.

EXPERIMENTAL

Materials

PMSF, GSH, NADPH, selenocystine and superoxide dismutase (Cu,Zn-SOD) were purchased from Sigma. Glutathione reductase was purchased from Boehringer-Mannheim GmbH. Enzyme-labelled antibodies, Balb/c mice and myeloma cells (NS-1) were supplied by the Institute of Military Veterinary Science of Changchun University. BSA and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Shanghai Institute of Biochemistry. Bio-Gel P-200, DEAE-52 and Ultrogel AcA54 were purchased from Bio-Rad, Whatman and LKB respectively. Xanthine oxidase (XOD) was purified from fresh milk [16]. Mitochondria were extracted from ox heart [17].

Induction of monoclonal antibodies

S-Substituted dinitrophenyl GSH (GSH-S-Dnp), used as the hapten, was synthesized as described previously [15,18]. It was coupled to BSA by glutaraldehyde [19] (Figure 1). The hapten has a maximum absorbance at 420 nm, and hapten density was determined by measuring A_{420} .

Monoclonal antibodies were raised against BSA-hapten conjugate using hybridoma methodology [15,20,21]. In brief, five Balb/c mice were injected intraperitoneally with BSA-hapten emulsified in adjuvant. After three rounds of immunization using BSA-hapten and adjuvant, mice were hyperimmunized with BSA-hapten in the absence of adjuvant and fused after 3 days. Spleen cells were fused with an NS-1 myeloma cell line in poly(ethylene glycol), and the hapten was used to screen hybridoma cells in an ELISA [22]. A total of three antibody-secreting hybridomas were isolated specifically for the hapten. All monoclonal antibodies were of the IgG class and propagated in ascites, purified by 45% $(\text{NH}_4)_2\text{SO}_4$ fractionation (4 °C,

Abbreviations used: cGPX, cytosolic glutathione peroxidase; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GPX, glutathione peroxidase; HX, hypoxanthine; MDA, malondialdehyde; Sec, selenocysteine; XOD, xanthine oxidase; Cu, Zn-SOD, superoxide dismutase; XPS, X-ray photoelectron spectroscopy.

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Figure 1 Synthesis of hapten, conjugate, antibody and selenium-containing catalytic antibody

a, SOCl_2 and 2,4-dinitrochlorobenzene, 0 °C; b, glutaraldehyde, 10 h; c, immunization and monoclonal antibody production; d, PMSF, 1 h; e, NaHSe, 40 °C, approx. 36 ~ 40 h.

pH 7.4), anion-exchange chromatography on DEAE-52 (5 mM Tris, pH 8.0, 20–500 mM NaCl, the antibody eluted in the 100 mM NaCl fraction), Bio-Gel P200 column chromatography (0.2 M Tris/0.2 M NaCl, pH 7.5) and affinity purification on a HiTrap protein G column (start buffer 20 mM sodium phosphate, pH 7.0; elution buffer 0.1 M glycine/HCl, pH 2.7). Antibodies (M_r 150000) were judged to be more than 95% homogeneous by SDS/PAGE under non-reducing conditions. Of the three catalytic IgGs, 4A4 showed significantly greater activity after chemical modification and was selected for further study. The Fab fragment (M_r 50000) of IgG was prepared by papain treatment and chromatography on HiTrap Protein G (Pharmacia).

Preparation of selenium-containing catalytic antibody (Se-4A4) and assays

Se-4A4 was prepared by chemically converting a reactive serine of antibody 4A4 into Sec with PMSF and sodium hydrogen selenide (NaHSe) [11,23]. Se-4A4 was isolated from the crude reaction mixture by Sephadex G-25 gel filtration and purified by affinity chromatography on thiopropyl-Sepharose 6B.

Reaction rates were measured by the coupled assay system in which oxidation of GSH is coupled to NADPH oxidation catalysed by glutathione reductase [24]. The reaction mixture consisted of 1 mM GSH, 0.25 mM NADPH, 1 unit of glutathione reductase, 0.5 mM H_2O_2 , 0.1 mM EDTA, 1 mM NaN_3 and 50 mM potassium phosphate buffer, pH 7.0. For the GPX activity, the rates were corrected for the background reaction between hydroperoxide and GSH. As a positive control for catalytic redox reaction, rabbit liver cGPX (85 units/mg) isolated in our laboratory was used [25]. The dissociation constants (K_d) of antibody–hapten (or GSH) complexes were measured by a competitive ELISA method [26]. Kinetic parameters and the inhibition by hapten were determined by Dalziel analysis [27]. To investigate the dependence of the rate on substrate concentration, the reaction rates were determined at several concentrations of H_2O_2 while keeping the concentration of GSH constant. The pH profile 5.7–11.2 and substrate specificity of Se-4A4 were obtained by directly monitoring the consumption of GSH or other thiol compound using the DTNB method (at 422 nm, 37 °C, pH 7.0 [28]).

The selenium content of the catalytic antibody and its fragments was determined by titration with DTNB [29] or by

inductively coupled plasma MS [30]. Selenium 3d (3/2, 5/2) electron signals were studied by X-ray photoelectron spectroscopy (XPS) with $\text{Al}_{K\alpha}$ as a target [31,32].

A model of free-radical damage to mitochondria was established using the hypoxanthine/xanthine oxidase (HX/XOD) system (0.2 mM HX, 0.3 mM EDTA, 0.1 mM FeCl_3 , 8×10^{-3} unit of XOD, 2.0 mg of mitochondria, 25 mM Hepes, pH 7.4, 25 °C). Protection of mitochondria by Se-4A4 from damage was determined by measuring the change in malondialdehyde (MDA) [33], a parameter reflecting lipid peroxidation. ESR signals of hydroxyl radical ($\cdot\text{OH}$) were obtained in the HX/XOD system in the presence and absence of Se-4A4 (1 mM GSH, 25 °C and 4.5 min), using 5,5'-dimethyl-1-pyrroline-1-oxide as a spin-trapping agent.

RESULTS

Hapten–BSA conjugate and monoclonal antibody

The synthesis of hapten, hapten–BSA conjugate and antibody 4A4 are outlined in Figure 1. The hapten–BSA conjugate gave hapten densities of 33 haptens per BSA (for M_r 84000). The conjugate was used to immunize Balb/c mice for the production of monoclonal antibodies. Monoclonal antibody 4A4 was shown by ELISA to bind to the hapten conjugated to BSA, and was cloned and injected into mice for production of ascites fluid. SDS/PAGE gave an M_r value of 150000. The dissociation constants for 4A4–hapten and 4A4–GSH were 2.0×10^{-8} and 5.6×10^{-8} M respectively (Table 1).

Se-4A4-catalysed reduction of H_2O_2 GSH

A rabbit IgG (rIgG) raised against human IgM($\text{Fc}\mu$) was also chemically modified by PMSF and NaHSe in the same way as IgG 4A4 (Figure 1). As shown in Table 2, 1.95 equivalents of selenol were incorporated per antibody 4A4 molecule, 0.95 equivalents per Fab fragment of Se-4A4, 0.05 equivalents per Fc

Table 1 Dissociation constants (K_d) for antibody–hapten (or substrate) complexes

rIgG, rabbit IgG.

Antibody–ligand	Dissociation constant (K_d) (M)
4A4–hapten	$(2.0 \pm 0.5) \times 10^{-8}$
Se-4A4–hapten	$(9.7 \pm 0.5) \times 10^{-8}$
4A4–GSH	$(5.6 \pm 0.7) \times 10^{-8}$
rIgG–GSH	$(4.8 \pm 0.6) \times 10^{-4}$

Table 2 Selenium contents of antibodies and fragments

Antibody	Sec amounts per protein molecule
4A4	0
rIgG	0
Se-4A4	1.95
Se-rIgG	1.84
Fab (Se-4A4)	0.95
Fc (Se-4A4)	0.05

Figure 2 Dalziel plot of Se-4A4 reaction with variable substrate H_2O_2 concentrations at two fixed concentrations of GSH in the physiological range

△, 1 mM GSH; ○, 2 mM GSH.

fragment and 1.84 equivalents per Se-rIgG molecule. The selenium 3d electron signals of Se-4A4, Se-rIgG and the natural cGPX determined by XPS are essentially identical, found in the 54.4 ± 0.3 eV region. Based on this information, the selenium in Se-4A4 and Se-rIgG is interpreted as not being bonded to oxygen but existing in the form of Sec, which is the same as that of the natural enzyme [31,32].

The relative efficiencies of Se-4A4, Se-rIgG and the natural GPX were investigated at pH 7.0 and 37 °C, as described in the Experimental section. Neither IgG 4A4 nor rIgG exhibit cGPX activity. The initial rate of the reaction (corrected for spontaneous oxidation in the absence of catalyst) was $2.6 \times 10^{-3} \text{ M} \cdot \text{min}^{-1}$ for Se-4A4 (0.2 μM), $1.5 \times 10^{-4} \text{ M} \cdot \text{min}^{-1}$ for Se-rIgG (0.2 μM), $1.2 \times 10^{-2} \text{ M} \cdot \text{min}^{-1}$ for rabbit liver cGPX (0.2 μM) and $2.1 \times 10^{-5} \text{ M} \cdot \text{min}^{-1}$ for the model compound ebselen (2 μM). The background rate, determined to be $3.66 \times 10^{-7} \text{ M} \cdot \text{min}^{-1}$, was used to calculate the uncatalysed rate constant ($k_{\text{uncat}} = 0.73 \text{ M}^{-1} \cdot \text{min}^{-1}$). Assuming that the rate has a first-order dependence on GSH concentration, these data indicate that Se-4A4 is at least 1238-fold more efficient than ebselen and 17-fold more efficient than Se-rIgG.

In order to probe the reaction mechanism, detailed kinetic studies were undertaken. Families of parallel Dalziel plots were constructed by holding GSH concentration constant while varying the concentration of H_2O_2 (Figure 2), which is consistent with a Ping Pong mechanism involving at least one covalent enzyme intermediate. The apparent kinetic parameters obtained from Figure 2 are summarized in Table 3.

The Se-4A4-catalysed reaction was monitored at several pH values (5.7, 6.6, 7.3, 7.9, 8.4, 8.8, 9.6, 10.2, 10.7, 11.2). The maximal rate occurs at $\text{pH} \approx 8.8$, which is the same as that of cGPX. The bell-shaped curve seen for $k_{\text{cat}}(\text{app})$ versus pH reflects the ionization of the enzyme-substrate complex, the decomposition of which is rate-limiting. The pH profile obtained for GPX activity of Se-4A4 is not significantly different from that of the natural cGPX. The similarity of the pH profiles of Se-4A4 and

Table 4 Effect of Se-4A4 on lipid peroxidation of mitochondria in HX/XOD system

Enzyme activity (units/ml)	Relative MDA content (%)
0 (Se-4A4)	100
11.9×10^{-3} (Se-4A4)	53.0
8.4×10^{-3} (Se-4A4)	71.8
1.1×10^{-5} (Se-4A4) + 800 (SOD)	69.2
0.1 (cGPX) + 800 (SOD)	70.3
0.1 (cGPX)	100
800 (SOD)	110

cGPX suggests that the same amino acids play key roles in the pH-dependence of GPX activity.

The Se-4A4-catalysed redox reaction is competitively inhibited by the hapten, characterized by a K_i of 5.7 μM . The competitive ELISA method gave a value of 97 nM for the K_d of the Se-4A4-hapten complex (Table 1).

Protective effect of Se-4A4 against radiation damage of mitochondria

Using the free-radical-damage system (HX/XOD), the protective effect of Se-4A4 on mitochondria was investigated. Mitochondrial damage by the free radical causes an increase in the level of MDA. The MDA level decreased when Se-4A4 was added to the HX/XOD system (Table 4). ESR spectra showed that the active oxygen in the HX/XOD system was $\cdot\text{OH}$ and the addition of Se-4A4 (1.4 nM) decreased $\cdot\text{OH}$ level by 76.1 %.

DISCUSSION

The idea that an antibody directed to a hapten (the transition-state analogue) which closely mimics the transition state for a given chemical reaction should catalyse that reaction has, together with hybridoma technology, proved fruitful in producing novel monoclonal antibodies that are enzyme-like catalysts. One of the most critical aspects in the overall process is the hapten design. All cGPXs are GSH-specific, and the triad, Sec, glutamine and tryptophan, is the cGPX catalytic centre, in which the selenium is activated by the proton-donating potential of co-ordinated glutamine and tryptophan residues. A Ping Pong mechanism, in which the active-site selenium of cGPX alternates between oxidized and reduced forms, was demonstrated. It is apparent that this bimolecular reaction is very complicated and proceeds through several intermediates which have not been fully elucidated. Therefore, not only the transition states for the reduction of H_2O_2 by GSH, but also the other factors required to achieve cGPX catalytic efficiency should be considered when the hapten is being designed. The other factors should have an antibody-binding pocket with specific affinity for GSH and the intrinsic chemical reactivity of the selenium prosthetic group,

Table 3 Kinetic parameters for the redox reaction of H_2O_2 with GSH catalysed by Se-4A4

$K_{\text{cat}}(\text{app})$ (min^{-1})	K_{uncat} ($\text{M}^{-1} \cdot \text{min}^{-1}$)	Φ_1 ($\text{M} \cdot \text{min}$)	Φ_2 ($\text{M} \cdot \text{min}$)	$K_m(\text{H}_2\text{O}_2)$ ($\Phi_1/\Phi_2[\text{GSH}]$)	$K_m(\text{GSH})$ ($\Phi_2/\Phi_1[\text{H}_2\text{O}_2]$)	$k_{\text{cat}}/K_m/k_{\text{uncat}}$
619.5	0.73	2.25×10^{-4}	2.50×10^{-3}	$8.99 \times 10^{-2} [\text{GSH}]$	11.1 $[\text{H}_2\text{O}_2]$	9.4×10^6

and the distance between Sec and the GSH-binding site should be within the active site. The hapten design may include elements of the substrate (GSH) so that selection is for substrate-specificity as well as for potential reactivity. However, using GSH itself as the hapten has some pitfalls: (1) the antigenicity of GSH is low; (2) GSH is unstable, as its thiol group is readily oxidized; (3) the antibody raised against GSH would have too high affinity for GSH to provide an effective space in the antibody-binding sites for Sec. S-Substituted dinitrophenyl GSH rather than GSH was therefore designed and synthesized as the hapten (Figure 1), and the above pitfalls were thereby removed. However, the active site of cGPX is a hydrophobic pocket located in a sizable depression on the surface [4,7]. The strategy of modifying GSH using a hydrophobic group (Dnp) could exploit not only the hydrophobic active site of an antibody containing the binding site of the substrate, but could also reduce the tight binding of GSH (Table 1).

As seen from the dissociation constants in Table 1, antibody 4A4 and Se-4A4 exhibited high affinity for GSH and the hapten, and antibody rIgG was found to have a very low affinity for GSH, suggesting that the binding pocket of antibody 4A4, rather than rIgG, has the GSH-specific binding site. This affinity, a major factor in catalysis, confers on Se-4A4 an approx. 17-fold higher cGPX activity than Se-rIgG (see the Results section). However, it is possible that the high affinity for GSH could also be the major reason why Se-4A4 ($k_{\text{cat}} = 619.5 \text{ min}^{-1}$) had a specific activity that was approx. 42% of that of the natural rabbit liver cGPX ($k_{\text{cat}} = 1445 \text{ min}^{-1}$). The major advantage of converting the reactive serine residue of protein into a Sec residue is that the three-dimensional structure of the protein is essentially the same as the unmodified protein [12]. This is also demonstrated by the similarity of the K_d values of 4A4 and Se-4A4 for the hapten.

Generally, there are more than ten serine residues in the antibody variable region. In the 4A4 variable region that contains the GSH-binding site, serine residues may be replaced by Sec. Analysis of the contents of the selenium-containing antibodies and fragments shows that approximately one serine residue is converted into Sec in each Fab of antibody Se-4A4 (Table 2). The above results suggest that the active-site geometry of antibody Se-4A4 is similar to that of the natural cGPX.

The Se-4A4-catalysed reaction was competitively inhibited by the hapten ($K_i = 5.7 \mu\text{M}$), demonstrating that the catalytic activity is associated with binding at the antibody-binding site. In an attempt to demonstrate that the modified serine is located in the binding site of the antibody, antibody 4A4 was preincubated with the hapten while chemical modification was carried out. The fact that serine modification does not occur, assessed by measuring the selenium content and reaction rates (described in the Experimental section), again confirms that the reactions are taking place in the antibody-binding site.

In addition to GSH, a number of other thiols have been examined as potential substrates for the peroxidase reaction catalysed by Se-4A4. Preliminary investigations on D,L-dithiothreitol, 2-mercaptoethanol, L-cysteine and N-acetylcysteine indicate that these thiols are not particularly good substrates for Se-4A4. The following relative activities were found at pH 7.0 and 37 °C (see the Experimental section): GSH, 100; dithiothreitol, 7; cysteine, 3; N-acetylcysteine, 3.5; 2-mercaptoethanol, 1. These results should not be surprising, since enzymes have evolved to bind specific substrates, and this specificity is one of their most desirable properties. 4A4 raised against the glutathione derivative (hapten) has a GSH-specific binding site (Table 1). Therefore, of all the thiol compounds tested, only GSH is able to serve as substrate for Se-4A4.

Scheme 1

As shown in Figure 2, the kinetic data at several GSH concentrations give characteristic parallel Dalziel plots, indicating a Ping Pong mechanism with at least one covalent intermediate, which is the same as the mechanism of cGPX. The apparent k_{cat} and $K_m(\text{H}_2\text{O}_2)$ values at 1 mM GSH, for example, were determined to be 619.5 min^{-1} and $90 \mu\text{M}$ respectively. Furthermore, a rate of acceleration ($k_{\text{cat}}/K_m/k_{\text{uncat}}$) of 9.4×10^6 was found. The kinetic data for both antibody Se-5A4 and cGPX can be fitted to a triple-transfer mechanism and may be described by the following general rate law [34]:

$$\frac{[\text{E}_0]}{\nu_0} = \frac{\Phi_1}{[\text{H}_2\text{O}_2]} + \frac{\Phi_2}{[\text{GSH}]}$$

where $[\text{E}_0]$ is the total enzyme concentration, and Φ_1 and Φ_2 are the kinetic coefficients. For antibody Se-4A4, acting on H_2O_2 and GSH at pH 7.0 and 37 °C, $\Phi_1 = 2.25 \times 10^{-4} \text{ M} \cdot \text{min}$ and $\Phi_2 = 2.50 \times 10^{-3} \text{ M} \cdot \text{min}$. The values of $K_m(\text{H}_2\text{O}_2)$ and $K_m(\text{GSH})$ may be expressed as $(\Phi_1/\Phi_2)[\text{GSH}]$ and $(\Phi_2/\Phi_1)[\text{H}_2\text{O}_2]$ respectively (Table 3). Under the same conditions, the K_d value of Se-4A4-hapten is smaller than the K_i value in the bimolecular reaction (see the Results section; Table 1). These results should not be surprising, since GSH and H_2O_2 were added when the K_i rather than K_d was being determined; this promoted the dissociation of the antibody-hapten complex. Although further experiments are needed to characterize each of the intermediates in the catalytic cycle, the turnover reaction may proceed via the mechanism shown in Scheme 1, which was originally proposed for cGPX.

In a comparison with the natural cGPX, the catalytic antibody Se-4A4 has some advantages. For example, the half life of Se-4A4 (pH 7.0; 0.05 M sodium phosphate buffer; 15 mg/ml; 4 °C) is 69 days, which is approx. two and half times as long as that of the natural rabbit liver cGPX. Furthermore, antibody Se-4A4 significantly protects cardiac mitochondria from active oxygen damage induced by the HX/XOD system (Table 4 and ESR result). The XOD-catalysed reaction is known to initially produce superoxide anions, which are spontaneously dismutated from H_2O_2 to $\cdot\text{OH}$ in the presence of Fe^{2+} . Therefore, Cu, Zn-SOD is not a good tool, because it enhances $\cdot\text{OH}$ generation in the reaction mixture (the MDA level was 110% of that of the control experiment). Natural cGPX is also not a good tool, because cGPX activity is destroyed by superoxide anions. In contrast with Cu, Zn-SOD and cGPX, antibody Se-4A4 significantly decreases the MDA level, and the use of Se-4A4 and SOD is more efficient than Se-4A4 alone. cGPX and Cu, Zn-SOD together inhibited 29.7% of MDA production, and the same protection was observed when the GPX activity of Se-4A4 was approx. 4 orders of magnitude less than that of the natural cGPX. The fact that the ESR signal ($\cdot\text{OH}$) declines by 76.1%

when Se-4A4 (1.4 nM) is added provides additional evidence for the above arguments.

Antibody Se-4A4 catalyses the reduction of H_2O_2 by GSH with a k_{cat} of 619.5 min^{-1} and a rate of acceleration ($k_{\text{cat}}/K_{\text{m}}/k_{\text{uncat}}$) of 10^7 . The catalytic efficiencies of GPX families differ from each other. For example, the value of k_{cat} for human plasma GPX [35] is 637.5 min^{-1} , which is almost the same as that of Se-4A4. Se-4A4 and the natural cGPX function via a similar catalytic mechanism. In addition, the stability of the antibody and protection of mitochondria against free radical are advantages over the natural cGPX. Therefore, this approach of preparing catalytic antibodies may yield useful catalysts for medical applications and also a greater understanding of the mechanism and evolution of biological catalysts.

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