Single Chain Antibody Displays Glutathione S-Transferase Activity*

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Substrate binding and the subsequent reaction are the two principal phenomena that underlie the activity of enzymes, and many enzyme-like catalysts were generated based on the phenomena. The single chain variable region fragment of antibody 2F3 (scFv2F3) was elicited against hapten GSH-S-DN2phBu, a conjugate of glutathione (GSH), butyl alcohol, and 1-chloro-2,4-dinitrobenzene (CDNB); it can therefore bind both GSH and CDNB, the substrates of native glutathione S-transferases (GSTs). It was shown previously that there is a serine residue that is the catalytic group of GST in the CDR regions of scFv2F3 close to the sulffhydryl of GSH. Thus, we anticipated that scFv2F3 will display GST activity. The experimental results showed that scFv2F3 indeed displayed GST activity that is equivalent to the rat θ-class GST T-2-2 and exhibited pH- and temperature-dependent catalytic activity. Steady-state kinetic studies showed that the apt values for the substrates are close to those of native GSTs, indicating that scFv2F3 has strong affinities for the substrates. Compared with some other GSTs, its apt value was found to be low, which could be caused by the similarity between the GSH-S-DN2phBu and the reaction product of GSH and CDNB. These results showed that our approach to imitating enzymes is correct, which is that an active site may catalyze a chemical reaction when a catalytic group locates beside a substrate-binding site of a receptor. It is important to consider product inhibition in hapten design in order to obtain a mimic with a high catalytic efficiency.

The generation of enzyme-like catalysts continues to be a fundamental goal for biochemists. Imitation of enzymes is helpful not only for understanding how enzymes work but also for obtaining practical catalysts. Natural enzymes catalyze biochemical reactions by binding one or two small molecules into an active site, with a catalytic group held nearby so as to interact effectively with the bound substrate molecule. Therefore, our opinion is that an active site may catalyze a chemical reaction when a catalytic group locates near a substrate-binding site of a receptor. Moreover, we have developed an approach to imitate enzymes, based on introducing a catalytic group into a receptor with a substrate-binding site to create an active site capable of catalyzing a chemical reaction. The ability to create novel active sites in this way permits us to systematically explore the basic principles of biological catalysis and to evaluate alternative catalytic pathways for particular reactions by comparing with native enzymes. So far, we have successfully generated some mimics with high glutathione peroxidase (GPX) activity by this approach (1–11).

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a superfamily of multifunctional enzymes involved in cellular detoxification by conjugating the tripeptide glutathione (GSH) to a wide range of endobiotic and xenobiotic electrophilic compounds (12–14). According to sequence, immunological, kinetic, and tertiary/quaternary structural properties, GSTs are grouped in at least 10 independent classes (15). In the active site of GSTs, the catalytic residue is typically either Tyr or Ser, presumed to act by inducing and stabilizing a reactive thiolate at the conjugating sulfur of the GSH substrate (14, 15).

To generate a substrate-binding site, one of the general strategies is standard monoclonal antibody preparation technique because the characteristic binding specificity of antibodies offers mimics the potential for unique substrate selectivity. The single chain variable region fragment of antibody (scFv) is a recombinant polypeptide consisting of a heavy chain variable region and a light chain variable region joined by a short peptide linker. It is the smallest antibody fragment that consistently maintains the binding specificity and affinity of the whole antibody (16).

The monoclonal antibody 2F3 was elicited against the hapten GSH-S-DN2phBu, which is a conjugate of GSH, butyl alcohol, and 1-chloro-2,4-dinitrobenzene (CDNB) (5). The single chain antibody scFv2F3, whose amino acid sequence is shown in Fig. 1, was obtained from monoclonal antibody 2F3 (6). Therefore, it can bind both GSH (17) and CDNB, which are substrates of native GSTs (Scheme 1). It was further converted to selenium-containing scFv2F3 (Se-scFv2F3) by chemical mutation (6). Molecular dynamics simulation studies of Se-scFv2F3 show that the chemical modification site of Se-scFv2F3 is Ser1652, which is close to the sulffhydryl of GSH (18). Thus, we anticipate the single chain antibody would display GST activity because it has both a substrate-binding site and a potentially catalytic group, which are collocated properly. Here we have reported the enzymic properties and kinetic behaviors of the scFv2F3 and discussed the importance of Ser as the catalytic residue of scFv2F3, as well as product inhibition and hapten design.

EXPERIMENTAL PROCEDURES

Materials—CDNB and cumene hydroperoxide (CuOOH) were obtained from Sigma. Glutathione reductase (type III baker’s yeast) was obtained from Roche Applied Science. NADPH was purchased from Roche Applied Science. Reduced glutathione and 1-bromo-4-nitrobenzen...
The concentration of protein was determined by the method of Lowry and coworkers (21), with crystalline bovine serum albumin as a standard.

The Mutation of Ser Residues in scFv2F3—Sodium hydroselenide was eluted against the hapten (5). Its V<sub>H</sub> and V<sub>L</sub> genes were cloned and sequenced from hybridoma 2F3 and used to construct the scFv2F3 by protein engineering.

Assay of Enzyme Activities—The specific activities of GST toward CDNB and 1-bromo-4-nitrobenzene were measured according to published methods (19). The activity of GST toward CDNB was measured in 50 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, 1 mM CDNB, and 1 mM GSH. The activity of GST toward 1-bromo-4-nitrobenzene was measured in 50 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, 0.1 mM 1-bromo-4-nitrobenzene, and 5 mM GSH. The reactions were conducted at 25 °C in a total volume of 0.7 ml. One unit of activity is defined as the amount of scFv2F3 that produces 1 μmol of product min<sup>-1</sup> at 25 °C. The specific activity is expressed in μmol min<sup>-1</sup> mg<sup>-1</sup> of protein.

The GPX activities were assayed as described with the coupled assay by Wilson et al. (20). The reactions were carried out in a total volume of 0.7 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, 1 unit of GSH reductase, and 10–50 mM protein. The mixture was preincubated for 7 min. Then 0.25 mM NADPH solution was added, and the mixture was incubated for 3 min at 37 °C. Thereafter, the reaction was initiated by the addition of 0.5 mM H<sub>2</sub>O<sub>2</sub> or 1.5 mM CuOOH. The activities were determined from the decrease of NADPH absorption at 340 nm. One unit of activity is defined as the amount of protein that catalyzes the turnover of 1 μmol NADPH/min. The specific activity is expressed in μmol min<sup>-1</sup> mg<sup>-1</sup> of protein.

The absorbance value was read on a UV-3100 spectrophotometer (Shimadzu). All initial rates were corrected for the background nonenzymatic reaction. The concentration of protein was determined by the method of Lowry et al. (21), with crystalline bovine serum albumin as a standard.

The Mutation of Ser Residues in scFv2F3—Sodium hydroselenide was prepared according to the method described (22). The mutation of Ser residues in scFv2F3 was performed according to Ref. 6. Briefly, 0.5 mg of scFv2F3 was dissolved in 2 ml of 50 mM sodium phosphate buffer, pH 7.0, 20 μl of phenylmethylsulfonyl fluoride solution (20 mg/ml acetonitrile) was added and incubated for 3 h at room temperature. The sulfonlated scFv2F3 was flushed using pure nitrogen for 20 min and then treated with 10 μl of 1 M sodium hydroselenide for 36 h at 35 °C. The crude reaction mixture was purified first by centrifugation and then loaded on a Sephadex G-25 column and eluted with buffer (20 mM Tris/HCl, pH 8.0). The active fractions were pooled and finally freeze dried to obtain Se-scFv2F3.

Determination of Optimal pH and Optimal Temperature for ScFv2F3 Catalysis—The optimal pH and optimal temperature of GST (CDNB) activity of scFv2F3 were measured with the same method as the GST (CDNB) activity assay. The initial rates were measured at the concentrations of 1 mM GSH and 1 mM CDNB. The pH value of buffer was changed in determining the optimal rates of the reaction to obtain the optimal pH condition for scFv2F3-catalyzed reaction. Similarly, the optimal temperature for scFv2F3-catalyzed reaction was determined at different temperatures.

Steady-state Kinetics of ScFv2F3—All kinetic experiments were performed in the same way as GST activity assay. The initial rates were measured by observing the increase of product absorption at 340 nm at several concentrations of one substrate while the concentration of the other substrate was fixed. GSH concentrations were varied from 0.1 to 1.0 mM; CDNB concentrations were also varied from 0.1 to 1.0 mM. The

TABLE 1

| Protein       | GST (CDNB) activity | GPX activity |
|---------------|---------------------|-------------|
|                | Units/mg            |              |
| GST 3–3       | 290.17              | 24           |
| Rat GST A     | 62                  | 25           |
| Human GST a   | 19                  | 25           |
| DmGSTS1–1     | 0.45                | 26           |
| rGST T2–2     | 0.9                 | 4            |
| ScFv2F3       | 0.997               |              |

* All values are the means of at least five determinations.
  * ND, no detectable GST or GPX activity.
  * The GST or GPX activities of rGST T2–2 and Se-rGST T2–2 are from Ref. 4.

TABLE 2

| Protein                | GST (CDNB) activity | GPX activity |
|------------------------|---------------------|-------------|
|                        | μmol/min/mg of protein |              |
| GST 3–3                | 290.17              | 24           |
| Rat GST A              | 62                  | 25           |
| Human GST a            | 19                  | 25           |
| DmGSTS1–1              | 0.45                | 26           |
| rGST T2–2              | 0.9                 | 4            |
| ScFv2F3                | 0.997               |              |

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The Mutation of Ser Residues in scFv2F3—Sodium hydroselenide was prepared according to the method described (22). The mutation of Ser residues in scFv2F3 was performed according to Ref. 6. Briefly, 0.5 mg of scFv2F3 was dissolved in 2 ml of 50 mM sodium phosphate buffer, pH 7.0, 20 μl of phenylmethylsulfonyl fluoride solution (20 mg/ml acetonitrile) was added and incubated for 3 h at room temperature. The sulfonlated scFv2F3 was flushed using pure nitrogen for 20 min and then treated with 10 μl of 1 M sodium hydroselenide for 36 h at 35 °C. The crude reaction mixture was purified first by centrifugation and then loaded on a Sephadex G-25 column and eluted with buffer (20 mM Tris/HCl, pH 8.0). The active fractions were pooled and finally freeze dried to obtain Se-scFv2F3.

Determination of Optimal pH and Optimal Temperature for ScFv2F3 Catalysis—The optimal pH and optimal temperature of GST (CDNB) activity of scFv2F3 were measured with the same method as the GST (CDNB) activity assay. The initial rates were measured at the concentrations of 1 mM GSH and 1 mM CDNB. The pH value of buffer was changed in determining the optimal rates of the reaction to obtain the optimal pH condition for scFv2F3-catalyzed reaction. Similarly, the optimal temperature for scFv2F3-catalyzed reaction was determined at different temperatures.

Steady-state Kinetics of ScFv2F3—All kinetic experiments were performed in the same way as GST activity assay. The initial rates were measured by observing the increase of product absorption at 340 nm at several concentrations of one substrate while the concentration of the other substrate was fixed. GSH concentrations were varied from 0.1 to 1.0 mM; CDNB concentrations were also varied from 0.1 to 1.0 mM. The
uncatalytic reaction affecting the measurement of the initial rate was taken into account and subtracted to obtain exact kinetic values. Kinetic data were analyzed by double reciprocal plotting.

RESULTS

Enzyme Activities of ScFv2F3—Using CDNB and 1-bromo-4-nitrobenzene as substrates we assayed the GST activities of scFv2F3, which were found to be 0.997 and 3.646 units/mg, respectively, and equivalent to the rat θ-class GST T-2-2 (rGST T2–2). The GPX activity of scFv2F3 toward CuOOH is 0.928 units/mg, but scFv2F3 did not exhibit GPX activity toward H2O2. However, after scFv2F3 converted into Se-scFv2F3 by chemical mutation, Se-scFv2F3 did not display any GST activity but exhibited a high GPX activity toward CuOOH or H2O2 (6), as did rGST T2–2. Its GST activity toward CDNB was 0.9 units/mg, and its GPX activity to H2O2 (4). The GST and GPX activities of scFv2F3 as well as rGST T2–2 were found to be 0.997 and 3.646 units/mg, respectively, and equivalent to the rat θ-class GST T-2-2 (rGST T2–2). The GPX activity of scFv2F3 was 0.928 units/mg, but scFv2F3 did not exhibit GPX activity toward H2O2. However, after scFv2F3 converted into Se-scFv2F3 by chemical mutation, Se-scFv2F3 did not display any GST activity but exhibited a high GPX activity toward CuOOH or H2O2 (6), as did rGST T2–2. Its GST activity toward CDNB was 0.9 units/mg, and its GPX activity to CuOOH was 2.1 units/mg, but it had no GPX activity to H2O2. After chemical mutation, rGST T2–2 was converted into Se-rGST T2–2. It then lost GST activity and gained a high GPX activity to CuOOH or H2O2 (4). The GST and GPX activities of scFv2F3 as well as rGST T2–2 are listed in Table 1.

Despite the diversity of substrates metabolized by this large family, CDNB is a relatively nonspecific GST reference substrate (23), and the GST (CDNB) activity of scFv2F3 catalyzed the conjugation of GSH with CDNB was determined as a function of substrate concentration, varying one substrate concentration while the other was fixed. Double reciprocal plots of the initial velocity versus substrate concentration showed a family of convergent lines for both substrates. The results are shown in Fig. 3, and the apparent kinetic parameters are listed in Table 3. With the concentration of CDNB increased from 0.1 to 1.0 mM, the apparent Michaelis constant \( K_{m\text{CDNB}} \) increased little, only from 0.00729 to 0.0166 mM, but the first-order rate constant \( k_{cat} \) increased obviously, from 5.604 to 13.670 min⁻¹, and the apparent second-order rate constant \( k_{cat}/K_{m\text{CDNB}} \) increased about one order of magnitude, from 20.36 to 125.9 min⁻¹ mM⁻¹, which are listed in Table 4.

The Optimal pH and Temperature for ScFv2F3-catalyzed Conjugation of GSH with CDNB—The GST (CDNB) activity of scFv2F3 was examined over the pH range from 4.5 to 11 and the temperature range from 15 to 60 °C (Fig. 2). For scFv2F3 catalyzing the conjugation of GSH with CDNB, the optimal pH was found to be 7.9 and the optimal temperature was found to be 44 °C. The GST (CDNB) activity of scFv2F3 at pH 6.5 is only 32.02% of that at pH 7.65. The activity of scFv2F3 at 25 °C is only 57.18% of that at 45 °C and at 60 °C only 5.58%. Above 55 °C it decreased rapidly, indicating that the structure of scFv2F3 is not steady under high temperatures.

Steady-state kinetics of ScFv2F3—The initial velocities catalyzed by scFv2F3 for the conjugation of GSH with CDNB were determined as a function of substrate concentration, varying one substrate concentration while the other was fixed. Double reciprocal plots of the initial velocity versus substrate concentration showed a family of convergent lines for both substrates. The results are shown in Fig. 3, and the apparent kinetic parameters are listed in Table 3. With the concentration of CDNB increased from 0.1 to 1.0 mM, the apparent Michaelis constant \( K_{m\text{GSH}} \) increased little, only from 0.2752 to 0.3325 mM, but the first-order rate constant \( k_{cat} \) increased obviously, from 5.604 to 13.670 min⁻¹, and the apparent second-order rate constant \( k_{cat}/K_{m\text{GSH}} \) increased about one order of magnitude, from 20.36 to 125.9 min⁻¹ mM⁻¹, which are listed in Table 4.

The same changes happened to scFv2F3, but the changes of substrate concentrations had almost no effect on \( K_{m\text{GSH}} \) and \( K_{m\text{CDNB}} \). The changes happened to \( K_{m\text{GSH}} \) and \( K_{m\text{CDNB}} \) while the concentrations of GSH increased. These parameters showed that the changes of substrate concentrations had almost no effect on \( K_{m\text{GSH}} \) and \( K_{m\text{CDNB}} \).

The following Equation 1 for the reciprocal velocity accounts for these plots

\[
\frac{[E]_0}{v_0} = \frac{\Phi_0}{[GSH]_0} + \frac{\Phi_{\text{GSH}}}{[CDNB]_0} + \frac{\Phi_{\text{CDNB}}}{[GSH]_0[CDNB]_0} + \frac{\Phi_{\text{GSH-CDNB}}}{[GSH]_0[CDNB]_0}
\]

where \( v_0 \) is the initial velocity of the enzymatic reaction, \( [E]_0 \) is the total concentration of scFv2F3, and \( \Phi_0, \Phi_{\text{GSH}}, \Phi_{\text{CDNB}}, \Phi_{\text{GSH-CDNB}} \) are Dalziel parameters (27), which are summarized in Table 4.

In Table 5, we compared the kinetic parameters for the GST (CDNB) activity of scFv2F3 with those of other native GSTs that have been
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**DISCUSSION**

ScFv2F3 displays GST activity, indicating that in scFv2F3 there is an active site that could bind the substrates GSH and CDNB and has a catalytic group to catalyze the conjugation of GSH to CDNB. *Km*GSH and *Km*CDNB of scFv2F3 are close to those of some native GSTs, indicating scFv2F3 has strong affinities to both GSH and CDNB. Then, which amino acid residue is the catalytic residue of scFv2F3? The experimental result showed that when scFv2F3 was converted into Se-scFv2F3 by chemical mutation of hydroxyl groups in the Ser residues of scFv2F3, it lost all GST activities and gained a high GPX activity to CuOOH or H2O2 (6), indicating that Ser in scFv2F3 could greatly contribute to GPX activity (4). Therefore, some Ser in scFv2F3 is the residue that can catalyze conjugation of GSH to electrophilic compounds.

Moreover, according to the three-dimensional structure of Se-scFv2F3 built by means of homology modeling, the chemical modification site of scFv2F3 is SerH52, and a hydrogen bond can be formed between the selenol group of the modified Ser and the sulfhydryl group of GSH (18). That is, the hydroxyl of the SerH52 is juxtaposed to the sulfhydryl group of GSH, and a certain chemical reaction can occur between them. Therefore, the 52nd amino acid residue is the catalytic residue of scFv2F3? Whether scFv2F3 exhibits GST or GPX activity depends on whether oxygen or selenium is present in the side chain of the 52nd amino acid residue. The difference of only one atom led to the catalytic group to catalyze the conjugation of GSH to CDNB.

The process of an enzymatic reaction usually is divided in three steps: binding its substrate, catalytic reaction, and releasing its product. A naturally occurring enzyme, after a long period of evolution, has formed a fine and ingenious system to accomplish catalysis. When an enzyme binds its substrate, its active site shape changes to create a shape into which the substrate fits. In the same way, the shape of the active site changes to release the product as soon as the product is produced. The rapid change of the shape of the active site ensures that an enzyme can bind the substrate and release the product continuously, which endows enzymes with great catalytic efficiency. However, most mimics are not as dynamic and flexible as native enzymes and do not have such a perfect system to recognize substrates and products. In particular, the shape of the active site of mimic cannot change continuously to bind its substrate and release its product.

In this experiment, the binding site of scFv2F3 induced by hapten GSH-S-DN2phBu has strong affinities to the two substrates and makes their active groups adjacent in space, which is advantageous to the conjugate reaction. In addition, fortunately there is a Ser as the catalytic residue of scFv2F3, which is juxtaposed to the sulfhydryl group of GSH. Therefore, scFv2F3 exhibits GST activities. This result indicates that our approach on imitating enzymes is correct. However, the catalytic

**TABLE 5**

| Species       | *k*cat | *K*mGSH | *K*mCDNB | *k*cat/*K*mGSH | *k*cat/*K*mCDNB | Ref. |
|---------------|--------|---------|----------|---------------|----------------|-----|
| ScFv2F3       | 2.286  | 0.4554  | 2.284    | 5.020         | 1.001          |     |
| GST 3–3       | 214.3  | ± 0.73  | 0.17     | ± 0.02        | 44.417         | 1244.97  |
| GST 5–5       | 260.2  | ± 6.4   | 0.37     | ± 0.08        | 1.65 ± 0.49    | 672.95  |
| GST6–6        | 234.4  | ± 2.4   | 1.75     | ± 0.43        | 2.88 ± 0.37    | 133.82 |
| GST8–8        | 216.9  | ± 4.8   | 2.20     | ± 0.27        | 0.86 ± 0.19    | 99    |
| Lucilia GST   | 53 ± 1 | 0.53 ± 0.041 | 0.165 ± 0.008 | 100 | 321 | 28 |

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capability of scFv2F3 and Se-scFv2F3 are much different, though the only difference between them is their catalytic groups. The GST activities of scFv2F3 are not high. On the contrary, Se-scFv2F3 showed a very high GPX activity, even much higher than native GPX from rabbit liver (6). The reasons are that GSH-S-DN2phBu and the reaction product of GSH and CDN2 are very similar and scFv2F3 binds the product too tightly to release it rapidly. However, neither the substrates nor the products in the reaction Se-scFv2F3 catalyzed are as similar as those in the reaction scFv2F3 catalyzed to GSH-S-DN2phBu; in the active site there is enough room for the substrates entering and the products leaving, which improves the catalytic velocity.

Therefore, it is important to consider product inhibition in hapten design in order to obtain an abzyme with a highly catalytic efficiency. Considering the affinity of antibody to antigen and the structure rigidity of abzymes, when we design a hapten to elicit a catalytic antibody we should consider not only its affinity to its substrate but also its dissociation with its product. By hapten redesign, a bigger active site could be obtained that will provide more room for product leaving from it to avoid product inhibition.

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