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Kinetics Study of a Selenium-Containing ScFv Catalytic Antibody That Mimics Glutathione Peroxidase

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The steady-state kinetics study and some enzymatic characterization of a selenium-containing scFv catalytic antibody (Se-scFv2F3) were carried out. A novel reaction formula of this abzyme-catalyzed reaction was proposed and a rate equation was obtained according to the formula. The constants in the equation were compared with Dalziel's parameters and the exact meanings of these constants were analyzed. The obtained kinetics parameters from the kinetics study of Se-scFv2F3 were analyzed and compared with those of native glutathione peroxidase. © 2001 Academic Press

Key Words: **scFv; glutathione peroxidase; selenium; kinetics; enzyme mimics.**

The generation of enzyme-like catalysts continues to be a fundamental goal for biochemists. The strategies employed in this area include the chemical synthesis of model systems (1–3) and the production of antibody molecules which bind carefully designed haptens (4, 5). The characteristic binding specificity of antibodies offers the potential for unique substrate selectivity by catalytic antibodies (abzyme). The ability to create novel active sites in this way (6) permits systematic exploration of the basic principles of biological catalysis and, through comparison with naturally occurring enzymes, evaluation of alternative catalytic pathways for particular reactions. Catalytic antibodies could have considerable value as biochemical or molecular-biological tools, as therapeutic agents, or in the synthesis of pharmaceuticals and novel materials.

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Single-chain variable region fragments of antibodies (scFv) are recombinant polypeptides composed of an antibody heavy-chain variable sequence (V_H) and a light-chain variable sequence (V_L) joined together by a short peptide linker. This protein is one of the smallest antibody fragments (7) that retain the ability to bind antigen. The linker ensures that both chains are expressed in equimolar amounts and increases the overall stability of the resulting protein as well (8, 9). Compared with monoclonal antibodies, scFv have several advantages including (a) bacterial expression, (b) small size for analysis by NMR or X-ray crystallography, and (c) ease of site-directed mutagenesis. One disadvantage is that scFv no longer contain an Fc portion to direct binding of a second antibody for immunoassays.

Glutathione peroxidase (GPX) is the first selenoenzyme to be detected in mammals. It is present in several tissues in either soluble or membrane-bound form in human (10–12), ox (13, 14), and sheep (15) erythrocytes as well as in human plasma (16) and placenta (12). Glutathione peroxidase is involved in the body's defense system, since it protects cells from oxidative damage (10, 11, 17, 18). The tetrameric molecule contains one seleno-cysteine molecule/subunit participating in the catalytic process (13). Selenium seems to be essential for glutathione peroxidase activity. Its role in the prevention of unsaturated fatty acid modification in some subcellular membranes after lipid peroxidation has been described by Tappel (12, 14).

The key kinetic properties of glutathione peroxidase have been elucidated and a number of plausible catalytic mechanisms suggested, although this mechanistic debate remains unsolved. Most researchers considered it to be ping-pong mechanism (19) while some other people considered it to be ordered sequential mechanism (20).

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A series of artificial enzymes including selenocyclodextrins (21–24) and selenium-containing antibodies (25–27) that mimic glutathione peroxidase have been developed by our group. A selenium containing scFv that mimic glutathione peroxidase (Se-scFv2F3) has just been obtained recently by gene engineering method by our group (28). In this article the scFv abzyme was studied with its steady-state kinetics behavior and some enzymatic characteristics. A novel reaction formula was proposed and a rate equation was thus written out and the meanings of the constants in the equation were discussed. According to the formula and rate equation, the kinetics parameters we obtained were analyzed and the differences between it and native glutathione peroxidase were discussed.

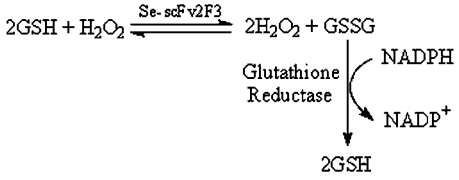
MATERIALS AND METHODS

Materials. Reduced glutathione (GSH) was obtained from Amresco. Oxidized glutathione (GSSG) was obtained from Roche. Glutathione reductase (type III baker's yeast), NADPH (tetrasodium salt) and cumene hydroperoxide (CuOOH) were obtained from Sigma. Hydroperoxide (H_2O_2), *tert*-butyl hydroperoxide (*t*-BuOOH), and sodium phosphate were obtained from Beijing Chemical Factory, China. All the other reagents were of analytic grade. The concentrations of hydroperoxides were determined by potassium permanganate titration method (29).

Acquisition of selenium-containing scFv. The acquire of selenium containing scFv has been described by our group (28). The serine of scFv was chemically mutated to be selenocysteine (Sec).

Determination of the association constant of selenium-containing scFv2F3 for GSH and GSSG. The association constant k_a of selenium containing scFv for GSH was determined at 30°C by measuring the intrinsic fluorescence intensity of the antibodies according to the previously method (30). The concentration of GSH or GSSG in the cuvette was increased step by step. The measurements were performed in an Hitachi 850 spectrophotofluorometer using λ_{ex} = 295. Binding parameters were determined as described previously (30).

Assay of kinetics of selenium-containing scFv2F3. The assay of kinetics of Se-scFv2F3 was similar to that of native GPX (31). The reaction was monitored by measuring the oxidized glutathione (GSSG) concentration resulting from the action of hydroperoxides on reduced glutathione (GSH) in the presence of glutathione reductase. The amount of oxidized glutathione produced was measured in terms of the decrease in the absorbance of NADPH at 340 nm ($\Delta^{\epsilon}_{NADPH-NADP}$ = 6.31 × 10^6 M^{-1} cm^{-1}). At the NADPH concentration used, the coupled reaction was a zero-order reaction with respect to NADPH and the oxidation of GSH was the limiting step. The oxidized glutathione produced in the first reaction was then reduced in the coupled one so that the reduced glutathione concentration was kept constant. The reaction is given in Scheme 1.



SCHEME 1

TABLE 1

Glutathione Peroxidase Activity of Se-scFv2F3, Se-2F3, Ebselen, and Native GPX ([GSH] = 1 mM, [H_2O_2] = 0.5 mM)

Species	GPX activity	
	U/μM	U/mg
Se-scFv2F3	2,380 ± 3%	79.3 ± 3%
Se-2F3	24,300 ± 3%	162.0 ± 3%
Ebselen	0.99	
Native GPX (rabbit liver)	5,780	85.7

To investigate the dependence of rate on substrate concentration, the initial rates were determined by following the decrease of NADPH absorption at 340 nm at several concentrations of one substrate while the concentration of the other substrate was kept constant. All kinetic experiments were performed in 0.5 ml of the reaction solution containing 50 mM potassium phosphate buffer, pH 7.0, 37°C, 1 mM EDTA, 1 unit of GSH reductase, 0.25 mM NADPH, appropriate concentration of GSH, H_2O_2, and Se-scFv2F3. The Se-scFv2F3 was preincubated with GSH, NADPH and GSH reductase. The reaction was initiated by addition appropriate concentrations of hydroperoxides. The non-enzymatic reaction affecting the measurement of the initial rate was taken into account and subtracted to obtain exact kinetic values.

Determination of optimal pH and optimal temperature for Se-scFv2F3-catalyzed reduction of hydroperoxides. Glutathione peroxidase activity of Se-scFv2F3 was measured with the same method as the kinetics study. The initial rates were measured using GSH and hydrogen peroxide at 1 and 0.5 mM, respectively. The pH value of the buffer was changed to determine the initial rates of the reaction to obtain the optimal pH condition for the Se-scFv2F3-catalyzed reaction. Similarly, the optimal temperature for the Se-scFv2F3-catalyzed reduction of hydroperoxide was also determined at a different temperature.

RESULTS AND DISCUSSIONS

Glutathione Peroxidase-like Activity of Se-scFv2F3

The activity of selenium containing scFv 2F3 catalyzed reduction of hydrogen peroxide by GSH is listed in Table 1. The activity is 2380 U/μM, which is about 41.2% of that of rabbit liver glutathione peroxidase. This is a relatively high figure, though it is only 9.8% of that of the intact monoclonal catalytic antibody 2F3. This activity is 2400 times that of the

TABLE 2

Association Constants for GSH of scFv before and after Chemical Mutation

Species	k_a
ScFv2F3	2.46 × 10^5
Se-scFv2F3	7.42 × 10^4

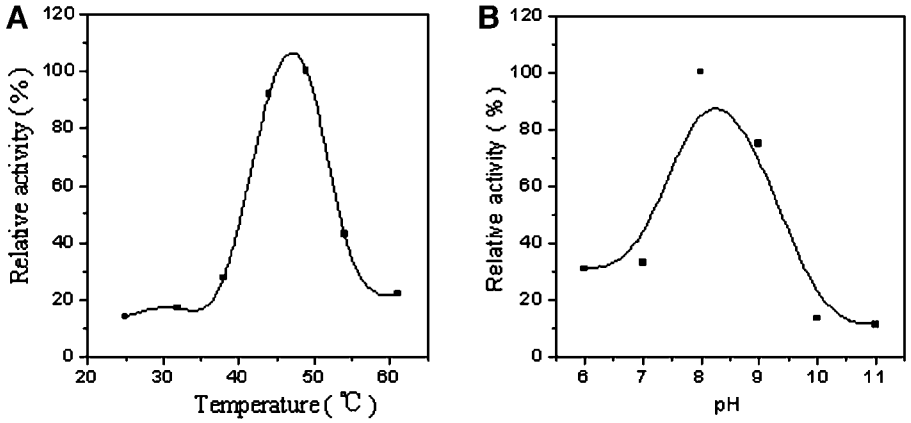


FIG. 1. The optimal temperature and pH for Se-scFv2F3-catalyzed reduction of hydrogen peroxide by GSH. (A) Optimal temperature. (B) Optimal pH.

well-studied glutathione peroxidase mimic ebselen (PZ 51).

Association Constant of Se-scFv2F3 for GSH

The association constants of scFv2F3 and Se-scFv2F3 for GSH were listed in Table 2. It could be seen that the association constant for GSH of scFv2F3 after chemical mutation was one order of magnitude lower than before mutation. This showed that the mutation of serine of the scFv affect the association of scFv for GSH, indicating that the serine mutated may has the function of binding to the substrate GSH molecule. Also this may be caused by the group and charge change induced by the mutation.

Optimal pH and Temperature for the Se-scFv2F3-Catalyzed Reduction of Hydrogen Peroxide by GSH

The relationship between the GPX activity and pH and temperature were in Fig. 1. The optimal pH for Se-scFv2F3-catalyzed reduction of hydrogen peroxide by GSH was found to be 8.27 and the optimal temperature was found to be 47.2°C. They are close to those of native glutathione peroxidase. The optimal pH and optimal temperature for native glutathione peroxidase are 8.8 and 50°C, respectively (32). As shown in Fig. 1, the GPX activity of Se-scFv2F3 at 37°C is only 28% that of at 42.7°C, and the activity at pH 7 is only 33% that of pH 8.3. It is obvious that the activity of the scFv abzyme was affected by pH and temperature very much. According to the fact that pH and temperature also affect the activity of native glutathione peroxidase, we think it may be a common phenomenon for selenium-containing enzyme with glutathione peroxidase activity.

Steady-State Kinetics Study of Se-scFv2F3

The initial rates catalyzed by Se-scFv2F3 for the reduction of hydroperoxide by GSH were determined

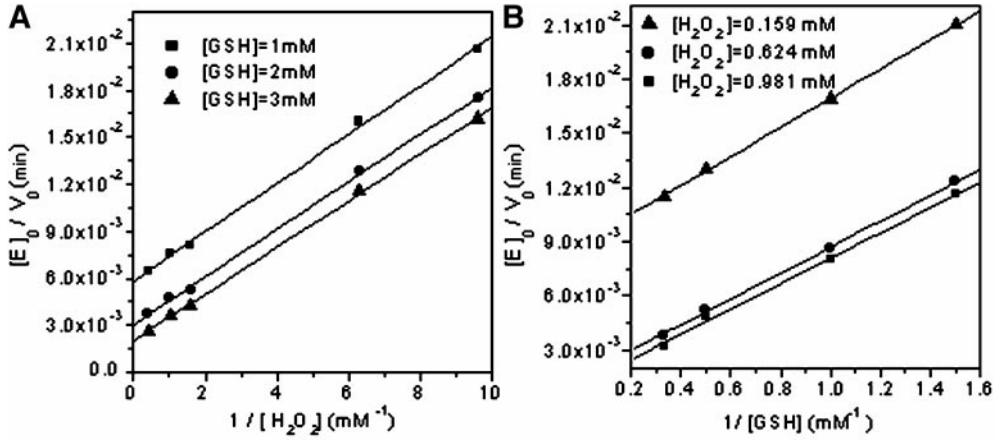


FIG. 2. Double-reciprocal plots for the reduction of H_2O_2 by GSH catalyzed by Se-scFv2F3. (A) $[E]_0/V_0$ vs $1/[H_2O_2]$. (B) $[E]_0/V_0$ vs $1/[GSH]$.

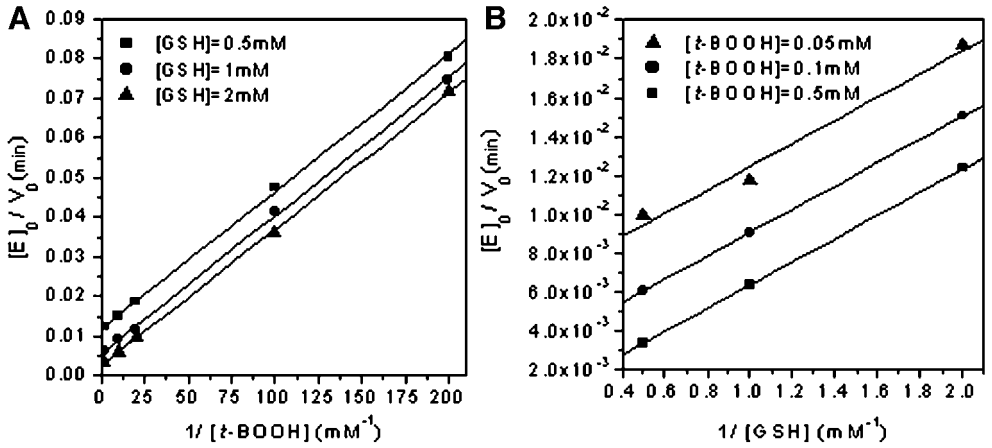


FIG. 3. Double-reciprocal plots for the reduction of *t*-BOOH by GSH catalyzed by Se-scFv2F3. (A) $[E]_0/V_0$ vs 1/[*t*-BOOH]. (B) $[E]_0/V_0$ vs 1/[GSH].

as a function of substrate concentration, varying one substrate concentration while the other fixed. Three hydroperoxide substrates were used in the study: hydrogen peroxide (H_2O_2), *tert*-butylhydroperoxide (*t*-BOOH), and cumene hydroperoxide (CuOOH). Double reciprocal plots of the initial velocity vs the concentration of the substrates yielded families of parallel lines for both substrates, which indicated that a ping–pong mechanism was involved. The plots were shown in Figs. 2, 3, and 4, respectively. The following equation for the reciprocal velocity accounts for these plots:

$$\frac{[E]_0}{V_0} = \Phi_0 + \frac{\Phi_G}{[GSH]} + \frac{\Phi_H}{[ROOH]},$$

where v_0 is the initial velocity of the enzymatic reaction, $[E]_0$ is the total abzyme concentration, and Φ_0, Φ_G, and Φ_H are Dalziel parameters (33). All the parameters were summarized in Table 3.

Discussion for the Parameters in the Rate Equation

The reaction catalyzed by the enzyme is in fact a three-molecule reaction though there are two substrates in the catalytic circle: hydroperoxides and GSH. This reaction is a *Bi Uni Uni Uni* ping–pong reaction according to Cleland's nomenclature method (34). The reaction process could be expressed in Scheme 2 according to Cleland's graphical presentation method (34). Needless to say the rate equation for this reaction is very complex and all the constants except k_1 and k_2 cannot be calculated.

In Eq. [1] we used is a simplified one in which all the constants could be calculated. To understand clearly the exact meaning of these constants we proposed a novel reaction formula, which was shown in Scheme 3

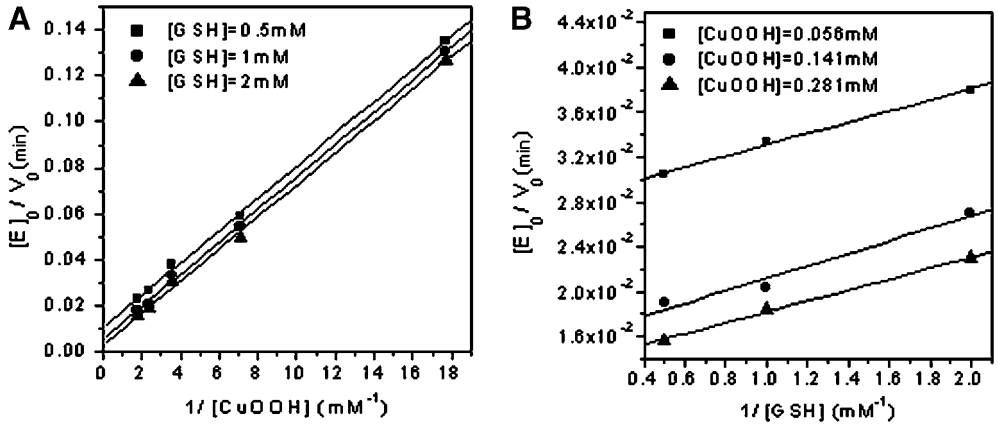
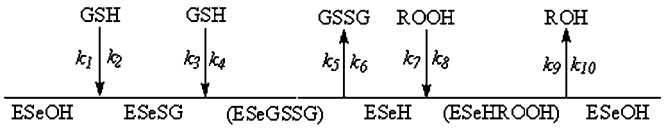


FIG. 4. Double-reciprocal plots for the reduction of CuOOH by GSH catalyzed by Se-scFv2F3. (A) $[E]_0/V_0$ vs 1/[CuOOH]. (B) $[E]_0/V_0$ vs 1/[GSH].



SCHEME 2

according to Fersht (35). The rate equation derived from this formula using the balance time method described by Fersht (35) is

$$\frac{[E]_0}{V_0} = \frac{k_{-1} + k_2}{k_1[\mathrm{GSH}]k_2} + \frac{1}{k_2} + \frac{1}{k_3[\mathrm{GSH}]} + \frac{1}{k_4} + \frac{1}{k_5[\mathrm{ROOH}]}.$$

Obviously, Eq. [2] and Eq. [1] are same in the form, which indicated that they are just the same one. By comparing the two equations we obtained, $\Phi_0 = 1/k_2 + 1/k_4$, $\Phi_H = 1/k_5$, and $\Phi_G = (k_{-1} + k_2)/k_1k_2 + 1/k_3$. From the formula in Scheme 3 it was found that k_1, k_{-1}, and k_3 are all constants in regards to the ability of the formation the enzyme–substrate intermediates from the original enzyme and substrates including the conjugation of the first and second GSH molecule. k_2 and k_4 are constants representing the velocity of the transformation of the enzyme–substrate intermediates to isomerase forms and products including the formation of ESeSG and ESeH and the release of ROH and GSSG. k_5 is the constant about the rates of the transform of ESeH to ESeOH oxidized by ROOH. So Φ_G is relevant to the formation of the two enzyme-substrate intermediates while Φ_H has the relationship to the ROOH induced oxidization of isomerase ESeH to ESeOH.

Φ_0 is the constant regarding the formation of isomerase from enzyme–substrate intermediates and the release of products. The constants we obtained showed that Φ_0 and Φ_G are similar for different hydroperoxide substrates, which was accordant with our previous judgment.

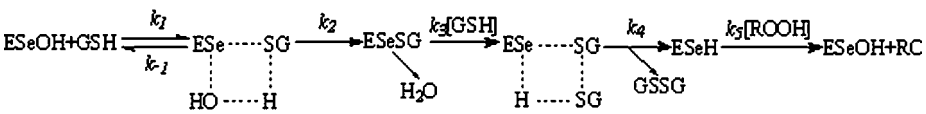
It is clear that this abzyme is different from native glutathione peroxidase for there is noΦ_0 in the kinetics parameters of native glutathione peroxidase (31). According to the balance time method the three items in the equation are reaction time of three reaction steps. The reaction time were 3.0×10^{-4}, 5.56×10^{-3}, and 5.96×10^{-3} min, respectively, for Φ_0, Φ_G, and Φ_H items with the concentration of GSH and H_2O_2 being 1 and 0.5 mM. Though Φ_0 is the least, it was only one order of magnitude lower than the other two items and can not be omitted. This is a very important difference between abzymes and native glutathione peroxidase. It was well known that there had no Φ_0 for the rate equation of native GPX given by Flohé (31). Φ_0 is not really nonexistent for native GPX but for the reason that the value is small enough to be omitted, meaning that the transformation of enzyme-substrate intermediates to isomerases and products for native GPX is very fast. For Se-scFv2F3 the time of this step could not be omitted which means that transformation of enzyme-substrate intermediate to isomerases and products is much slower than that of native GPX.

TABLE 3

Kinetics Parameters of Selenium-Containing scFv2F3-Catalyzed Reduction of Different Hydroperoxide Substrates by GSH

Hydroperoxide substrate species	Φ_0 (min)	Φ_H (M · min)	Φ_G (M · min)
Hydrogen peroxide	3.0×10^{-4}	2.98×10^{-6}	5.56×10^{-6}
tertial-Butyl hydroperoxide	3.35×10^{-4}	6.86×10^{-7}	5.72×10^{-6}
Cumene hydroperoxide	3.2×10^{-4}	1.41×10^{-6}	5.61×10^{-6}

The reason for the difference between abzyme and native GPX is, as we believe, that abzyme has no excellent steric structure of native GPX to make the whole reaction process at high rates. The hapten of monoclonal antibody 2F3 is GSH derivative and thus the conjugation of the abzyme to the first GSH molecule may be fast but the rates of other steps such as the conjugation of the second GSH molecule may be quite slow. This is also a general problem for catalytic antibodies. In fact for generating catalytic antibodies, which could be compared to native enzymes it is not enough just focusing on hapten design. New methods must be developed to induce more accurate 3D structure into the binding site of antibodies. The methods could be chemical or molecular biological in nature. All these works depend on the more knowledge of enzymatic structure, enzyme–substrate interaction, and the exact mechanism of the enzyme-catalyzed reaction.



SCHEME 3

REFERENCES

1. Breslow, R. (1991) *Ciba Found. Symp.* **158,** 115–127.
2. Cram, D. J. (1988) *Science* **240,** 760–767.
3. Lehn, J. M. (1985) *Science* **227,** 849–856.
4. Lerner, R. A., Benkovic, S. J., and Schultz, P. G. (1991) *Science* **252,** 659–667.
5. Lewis, C. T., and Hilvert, D. (1991) *Curr. Opin. Struct. Biol.* **1,** 624–629.
6. Jencks, W. P. (1969) *in* Catalysis in Chemistry and Enzymology, p. 228, McGraw Hill, New York.
7. Givol, D. (1991) *Mol. Immunol.* **28,** 1379–1386.
8. Glockshuber, R., Malia, M., Pfitzinger, I., and Pluckthun, A. (1990) *Biochemistry* **29,** 1362–1367.
9. Pantoliano, M. W., Bird, R. E., Johnson, S., Asel, E. D., Dodd, S. W., Wood, J. F., and Hardman, K. D. (1991) *Biochemistry* **30,** 10117–10125.
10. Mills, G. C. (1957) *J. Biol. Chem.* **229,** 589–599.
11. Mills, G. C., and Randall, H. P. (1957) *J. Biol. Chem.* **229,** 189–197.
12. Tappel, A. L. (1965) *Fed. Proc.* **24,** 73–78.
13. Flohé, L., Günzler, W. A., and Schock, H. H. (1973) *FEBS Lett.* **32,** 132–134.
14. Tappel, A. L. (1967) *in* Selenium in Biomedicine (Muth, O. H., Oldfield, J. E., and Weswig, P. H., Eds.), pp. 345–361, AVI Publishing, Westport, CT.
15. Oh, S. H., Ganther, H. E., and Hoekstra, W. G. (1974) *Biochemistry* **13,** 1825–1828.
16. Broderick, D. J., Deagen, J. T., and Whanger, P. D. (1987) *Inorg. Biochem. J.* **30,** 299–308.
17. Reddy, C. C., Tu, C. P. D., Burgess, J. R., Ho, C. Y., Scholz, R. W., and Massaro, E. J. (1981) *Biochem. Biophys. Res. Commun.* **101,** 970–978.
18. Flohé, L. (1988) *Basic Life Sci.* **49,** 663–668.
19. Flohé, L. (1989) *in* Coenzymes and Cofactors: Glutathione. Chemical, Biochemical, and Medical Aspects (Dolphin, D., Pwlson, R., and Avramon, O., Eds.), pp. 644–731, Wiley, New York.
20. Carsol, M. A., Sonaglia, I. P., Lesgards, G., Mouren, G. M., Puigserver, A., and Santimone, M. (1997) *Eur. J. Biochem.* **247,** 248–255.
21. Ren, X. J., Liu, J. Q., Luo, G. M., Zhang, Y., Luo, Y. M., Yan, G. L., and Shen, J. C. (2000) *Bioconjugate Chem.* **11,** 682–687.
22. Ren, X. J., Yang, L. Q., Liu, J. Q., Su, D., You, D. L., Liu, C. P., Zhang, K., Luo, G. M., Mu, Y., Yan, G. L., and Shen, J. C. (2000) *Arch. Biochem. Biophys.* **387,** 250–256.
23. Liu, J. Q., Luo, G. M., Ren, X. J., Mu, Y., Bai, Y., and Shen, J. C. (2000) *Biochim. Biophys. Acta* **1481,** 222–228.
24. Liu, J. Q., Gao, S. J., Luo, G. M., Yan, G. L., and Shen, J. C. (1998) *Biochem. Biophys. Res. Commun.* **247,** 397–400.
25. Luo, G. M., Zhu, Z. Q., Ding, L., Gao, G., Sun, Q. A., Liu, Z., Yang, T. S., and Shen, J. C. (1994) *Biochem. Biophys. Res. Commun.* **198,** 1240–1247.
26. Zhu, Z. Q., Ding, L., Luo, G. M., Liu, Z., Sun, Q. A., Yang, T. S., and Shen, J. C. (1994) *Biochem. Biophys. Res. Commun.* **202,** 1645–1650.
27. Ding, L., Liu, Z., Zhu, Z. Q., Luo, G. M., Zhao, D. Q., and Ni, J. Z. (1998) *Biochem. J.* **332,** 251–255.
28. Ren, X. J., Gao, S. J., Huang, H. L., Liu, Z., Mu. Y., Liu, J. Q., You, D. L., Yan, G. L., Luo, G. M., Yang, T. S., and Shen, J. C. (2001) *Biochem. J.*, in press.
29. Kolthoff, I. M., and Medalia, A. I. (1949) *J. Am. Chem. Soc.* **71,** 3789–3792.
30. Jolley, M. E., and Glaudemans, C. P. J. (1974) *Carbohydr. Res.* **33,** 377–382.
31. Flohé, L., Loschen, G., Günzler, W. A., and Eichele, E. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353,** 987–999.
32. Lerner, R. A., and Benkovic, S. J. (1990) *Chemtracts—Org. Chem.*, 1–36.
33. Dalziel, K. (1957) *Acta Chem. Scand.* **11,** 1706–1723.
34. Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67,** 104–137.
35. Fersht, A. (1984) Enzyme Structure and Mechanism, 2nd ed., Freeman, New York.