

Is thioredoxin the physiological vitamin K epoxide reducing agent?

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E. coli thioredoxin plus thioredoxin reductase have previously been shown to replace dithiothreitol as the electron donor for mammalian liver microsomal vitamin K epoxide reduction in vitro. Such activity is dependent on detergent disruption of the microsomal membrane integrity. A previously characterized salicylate-inhibitable pathway for electron transfer from endogenous cytosolic reducing agents to the microsomal epoxide reducing warfarin-inhibitable enzyme is not inhibited by known alternate substrates and inhibitors of the thioredoxin system nor by antibodies against thioredoxin.

Vitamin K epoxide reductase; Thioredoxin; Thioredoxin reductase; Salicylate inhibition

1. INTRODUCTION

The metabolic transformations of vitamin K, hydroquinone to epoxide and regeneration of the hydroquinone via the quinone, and their relation to vitamin K-dependent protein synthesis have been well established [1]. The reactions of vitamin K epoxide and vitamin K quinone reduction have been shown to be the sites of warfarin anticoagulant action. However, the physiologically relevant reducing agent for these reaction, replaced by dithiothreitol (DTT) in vitro, has not been identified.

A salicylate-inhibitable pathway for the transfer of reducing equivalents from an endogenous cytosolic donor(s) to the epoxide-reducing, warfarin-inhibitable microsomal catalytic center has been demonstrated by Hildebrandt and Suttie [2]. Carlisle and Suttie [3] previously demonstrated that the DTT-dependent vitamin K epoxide reductase is located on the luminal surface of the rough endoplasmic reticulum (RER). An as yet ill-defined mechanism for transfer of reducing equivalents across the microsomal membrane, therefore, presumably exists.

Vermeer and coworkers [4] demonstrated that *E. coli* thioredoxin plus its reductase could drive either vitamin K or vitamin K epoxide-dependent peptide (and protein) carboxylation in partially purified solubilized microsomal system. Similar results were apparently obtained with intact microsomes, but no data were provided.

Silverman and Nandi [5] demonstrated directly by HPLC assay that *E. coli* thioredoxin plus its reductase

are capable of driving microsomal vitamin K epoxide reduction in both solubilized and intact rat and beef liver microsomes. As shown here, the results in intact microsomes are dependent on the concentration of detergent in which the vitamin is added, and thioredoxin is probably not the physiologically relevant reducing agent for this system in vivo.

2. EXPERIMENTAL

2.1. Preparations

Whole rat liver microsomes and cytosol in 0.25 M sucrose, 0.01 M Tris-HCl, 1 mM EDTA, pH 7.6 buffer (Buffer A) were prepared immediately before use (within two hours) from perfused livers of freshly killed rats as previously described [6]. All assays were conducted in Buffer A at room temperature.

2.2. Assays of thioredoxin–thioredoxin reductase and anti-thioredoxin

Purified *E. coli* thioredoxin obtained from Calbiochem was dissolved in Buffer A to yield a stock solution of 5 mg/ml (0.43 mM). Purified crystalline *E. coli* thioredoxin reductase in ammonium sulfate suspension (American Diagnostica) was diluted with Buffer A to a concentration of 0.35 mg/ml (4.7 μ M). Bovine pancreatic insulin, 24.5 iU/mg from Sigma was dissolved to yield a stock solution of 10 mg/mL (1.6 mM). Activity of thioredoxin reductase was assayed as insulin-dependent NADPH consumption monitored by absorbance at 340 nm, as described by Holmgren [7], and was found to be 3.6 μ mol NADPH consumed/min/mg thioredoxin reductase. Under conditions where the concentration of thioredoxin was limiting, the specific activity of the Calbiochem thioredoxin was 0.426 μ mol NADPH consumed/min/mg. Goat anti-*E. coli* thioredoxin serum (American Diagnostica) was found to have a titer equivalent to 190 μ g thioredoxin neutralizing units/ml serum based on inhibition of activity.

The amount of thioredoxin present in freshly prepared rat liver cytosol corresponded to a NADPH consumption rate equivalent to 0.18 mg/ml of *E. coli* thioredoxin. Activity was inhibited 65% by addition of 20 μ l anti-thioredoxin serum/0.1 ml of cytosol and almost completely by 40 μ l/0.1 ml of cytosol.

2.3. Assays of vitamin K epoxide reductase

For assays of thioredoxin-dependent vitamin K epoxide reduction,

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incubations containing 0.1 ml of microsomes in Buffer A (equivalent to 0.5 g liver/ml), 5 μ l of 1 mM vitamin K epoxide in 1% Emulgen 911, plus or minus an additional 20 μ l of 1% Emulgen 911, 40 μ l of thioredoxin (5 mg/ml), and 50 μ l of 20 mM NADPH, plus or minus inhibitor or vehicle, were assembled on ice in a total volume of 0.5 ml of Buffer A. Following a two-minute thermal equilibration to room temperature, the reactions were initiated with 10 μ l of thioredoxin reductase (0.35 mg/ml) and incubated for 10 min. No reaction was observed for the thioredoxin system alone, or in the absence of any components of the complete system. For assays of the dithiothreitol-dependent reaction, a 1 mM final concentration of DTT was used in place of the thioredoxin/reductase/NADPH system. Addition of the complete thioredoxin system did not alter the activity observed with DTT alone. For assays of cytosol-dependent vitamin K epoxide reduction, aliquots of 0.5 ml of microsomes (equivalent to 1 g liver/ml) resuspended in 0.5 g liver/ml of fresh cytosol, plus or minus the indicated inhibitors, were assembled on ice. Following a two-minute thermal equilibration, reactions were initiated by the addition of 5 μ l of 1 mM vitamin K epoxide in 1% Emulgen 911 and incubated for 30 min at room temperature. In all cases, the reactions were stopped by addition of 0.5 ml of 2 mM HgCl₂, extracted with 2 ml of isopropanol/hexane (1:1), and the amount of vitamin K formed quantitated by reverse-phase HPLC with UV detection, as previously described [6].

3. RESULTS

Initial attempts to reproduce the thioredoxin-dependent reduction of vitamin K epoxide in whole microsomes were unsuccessful. When the vitamin was added in a low final concentration of detergent (0.01% Emulgen 911) as was customary in this laboratory, thioredoxin-dependent reduction was almost negligible (Table I). When additional detergent was added to yield the appropriate final concentration used in the work of Silverman and Nandi [5], comparable rates of reduction were observed using either reductant. The decrease in the DTT-dependent rate at high detergent concentration is typical of many detergents.

Under conditions where thioredoxin-dependent vitamin K epoxide reduction was significant (at 0.05% Emulgen 911), the reaction was no more sensitive to salicylate inhibition than was the DTT-dependent reaction. Inhibition at 200 μ M salicylate was minimal (Table I). In contrast, when microsomal vitamin K epoxide reduction was assayed in the presence of freshly prepared cytosol as the source of reducing agent, the reaction was inhibited 60 percent by 200 μ M salicylate

Table I

Vitamin K epoxide reduction: effects of reductant, detergent and salicylate^a

| Incubation conditions | Reductant | |
|---------------------------|-----------|-------------|
| | DTT | Thioredoxin |
| 0.01% Emulgen | 1.98 | 0.023 |
| 0.05% Emulgen | 0.42 | 0.43 |
| + 200 μ M Salicylate | 0.35 | 0.35 |
| + 1000 μ M Salicylate | 0.21 | 0.18 |

^a nmol K formed/incubation. Average for duplicate incubations differing by less than 10 percent. See section 2 for details.

(Table II). These results are consistent with a weak direct effect of salicylate on the microsomal vitamin K epoxide reducing center, itself, and suggest that thioredoxin, like DTT, bypasses the more sensitive salicylate inhibitable site involved in the endogenous reductant electron transfer pathway [2].

Table II compares the effects of various substrates and inhibitors on: (a) microsomal vitamin K epoxide reductase activity assayed using cytosol as the source of reducing equivalents, and (b) cytosolic thioredoxin system activity (assayed as insulin-dependent NADPH consumption). Salicylate inhibited vitamin K epoxide reduction but did not inhibit the cytosolic thioredoxin system. This was also confirmed for the purified *E. coli* system components. Actinomycin (doxorubicin) and mitomycin [8] and NADP⁺ [9] inhibited the thioredoxin system by about 50 percent as expected from the references cited, but did not inhibit vitamin K epoxide reduction. Insulin, a substrate for the thioredoxin system, did not inhibit cytosol dependent vitamin K epoxide reduction as might be expected if both competed for limiting thioredoxin. Alloxan, a substrate for thioredoxin reductase [10], did inhibit cytosol-dependent vitamin K epoxide reduction (data not shown). However, alloxan also inhibited the DTT-dependent reaction and could be shown to titrate the amount of disulfide present. Finally, and most significantly, goat anti-*E. coli* thioredoxin antiserum was shown to inhibit thioredoxin activity in rat liver cytosol under conditions where it did not inhibit cytosol-dependent vitamin K epoxide reduction.

4. DISCUSSION

Several lines of evidence suggest that both vitamin K epoxide reduction and vitamin K quinone reduction are catalyzed by the same microsomal enzyme [11,12] via a ping-pong kinetic mechanism [6,11], probably involving an active site disulfide [13]. Evidence suggests that coumarin anticoagulants, such as warfarin, act by blocking the reduction of the active site disulfide [13,14].

Table II

Effects of inhibitors on cytosol-dependent microsomal vitamin K epoxide reduction and on the cytosolic thioredoxin-thioredoxin reductase system^a

| Addition | Epoxide reduction | Thioredoxin activity |
|--|-------------------|----------------------|
| Salicylate (200 μ M) | 38 | 100 |
| Actinomycin (100 μ M) | 96 | 53 |
| Mitomycin (100 μ M) | 107 | 38 |
| NADP ⁺ (100 μ M) | 102 | 34 |
| Insulin (1 mg/mL) | 100 | — |
| Anti-thioredoxin (40 μ l/0.1 ml cytosol) | 91 | 6 |

^a Percent of control activities: 0.165 nmol vitamin K formed/incubation and 0.163 AU/min at 340 nm as described in section 2. Average of duplicates differing by less than 10 percent.

Previous work [2] has established a salicylate inhibitable pathway for the transfer of reducing equivalents from cytosol to the epoxide reducing center. This pathway is bypassed by dithiothreitol. The identity of the electron carriers in this pathway and the site of action of salicylate (in addition to its weaker effect at a site not bypassed by DTT) remain to be determined. The evidence presented here suggest that the thioredoxin system is not part of this pathway.

Disulfhydryl reagents, such as dithiothreitol, but not most other general or physiological reducing agents, are able to drive both the reduction of vitamin K epoxide and vitamin K quinone by liver microsomes [13]. Reduced thioredoxin is able to act in this capacity [5] and the kinetic mechanism (ping pong) is the same as for dithiothreitol [6]. However, the evidence presented here suggests that the thioredoxin-dependent reaction is not likely to be physiologically relevant. Thioredoxin is predominantly a soluble cytoplasmic protein that also has some association with membranous structures [15,16], however, clear evidence of its presence on the inside of the RER has not been presented. It is not apparent that thioredoxin in intact cells would have access to the epoxide reducing enzyme which has been localized on the luminal surface of intact RER membranes [3]. Further, the absence of a thioredoxin-dependent reaction at a low (0.01%) detergent concentration suggests that there is not a mechanism for transfer of reducing equivalents from this source across intact RER membranes. In the experiments of Vermeer and coworkers [4] on thioredoxin plus vitamin K-or vitamin K epoxide-dependent carboxylation, the vitamin was apparently added in a mixture of phospholipid and sodium cholate. Conceivably, the final level of cholate (non clearly stated) was sufficient to permeabilize the microsomal membranes of their 'intact' system enough to permit entry of thioredoxin.

Other thioredoxin like molecules are clearly RER resident proteins (i.e. protein disulfide isomerase [17] and ERp72 [18]). Recently, Soute, et al. [19] showed that purified protein disulfide isomerase stimulates thioredoxin-dependent vitamin K-dependent carboxylase and vitamin K epoxide reductase activities in a solubilized microsomal preparation. This paper raised the interesting suggestion that protein sulfhydryl groups of nascent secretory proteins could provide a source of reducing equivalents for vitamin K epoxide and quinone reduction. Further work is needed to test the thioredoxin requirement of this reaction (activity was tested with reduced RNase as the reductant, in the presence of

thioredoxin, plus protein disulfide isomerase, but not in the absence of thioredoxin) and to test the involvement of protein disulfide isomerase in intact membrane systems and cells more closely resembling the situation in-vivo.

Thioredoxin is known to be active in a variety of biochemical oxidation reduction reactions of physiological interest (e.g. the ribonucleotide reductase reaction) and in others as a useful but somewhat arbitrary reagent (e.g. in-vitro protein refolding). Regardless of its physiological relevance in vitamin K function, thioredoxin may be a useful reagent in the study of vitamin K epoxide reductase (e.g., studies of inhibitors which are unstable in the presence of excess thiol reagents).

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REFERENCES

- [1] Suttie, J.W. (1988) *Current Advances in Vitamin K Research*, Elsevier, Amsterdam.
- [2] Hildebrandt, E. and Suttie, J.W. (1984) *J. Pharm. Pharmacol.* 36, 586-591.
- [3] Carlisle, T.L. and Suttie, J.W. (1980) *Biochemistry* 19, 1161-1167.
- [4] Van Haarlem, L.J.M., Soute, B.A.M. and Vermeer, C. (1987) *FEBS Lett.* 222, 353-357.
- [5] Silverman, R.B. and Nandi, D.L. (1988) *Biochem. Biophys. Res. Commun.* 155, 1248-1254.
- [6] Hildebrandt, E.F., Preusch, P.C., Patterson, J.L. and Suttie, J.W. (1984) *Arch. Biochem. Biophys.* 228, 480-492.
- [7] Holmgren, A. (1984) *Methods Enzymol.* 107, 295-300.
- [8] Mau, B.-L. and Powis, G. (1990) *Free Rad. Res. Commun.* 8, 365-372.
- [9] Williams, C.H., Jr., in: *The Enzymes*, 3rd Ed., vol. 13, (P.D. Boyer, Ed.), Academic Press, New York, 1976, pp. 142-148.
- [10] Holmgren, A. and Lyckenborg, C. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5149-5152.
- [11] Preusch, P.C. and Smalley, D.M. (1990) *Free Rad. Commun.* 8, 401-415.
- [12] Gardill, S.L. and Suttie, J.W. (1990) *Biochem. Pharmacol.* 40, 1055-1061.
- [13] Lee, J.J., Principe, L.M. and Fasco, M.J. (1985) *Biochemistry* 24, 7063-7070.
- [14] Fasco, M.J., Principe, L.M., Walsh, W.A. and Friedman, P.A. (1983) *Biochemistry* 22, 5655-5660.
- [15] Holmgren, A. and Luthman, M. (1978) *Biochemistry* 17, 4071-4077.
- [16] Rozell, B., Hansson, H.A., Luthman, M. and Holmgren, A. (1985) *Eur. J. Biol.* 38, 79-86.
- [17] Freedman, R.B. (1989) *Cell* 57, 1069-1072.
- [18] Mazzarella, R.A., Srinivasan, M., Haugejorden, S.M. and Green, M. (1990) *J. Biol. Chem.* 265, 1094-1101.
- [19] Soute, B.A.M., Groenen-van Dooren, M.C.L., Holmgren, A., Lundstrom, J. and Vermeer, C. (1992) *Biochem. J.* 281, 255-259.