BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 268–274 (1996)
ARTICLE NO. 1165

Efficient Reduction of Lipoamide and Lipoic Acid by Mammalian Thioredoxin Reductase

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Received June 27, 1996

Reduction of the antioxidant lipoic acid has been proposed to be catalyzed *in vivo* by lipoamide dehydrogenase (LipDH) or glutathione reductase (GR). We have found that thioredoxin reductase (TR) from calf thymus, calf liver, human placenta, and rat liver efficiently reduced both lipoic acid and lipoamide with Michaelis-Menten type kinetics in NADPH-dependent reactions. In contrast to LipDH, lipoic acid was reduced almost as efficiently as lipoamide. Under equivalent conditions at 20°C, pH 8.0, mammalian TR reduced lipoic acid by NADPH 15 times more efficiently than the corresponding NADH dependent reduction catalyzed by LipDH (297 min^{-1} for TR vs. 20.3 min^{-1} for LipDH). Moreover, TR was 2.5 times faster in reducing lipoic acid with NADPH than in catalyzing the reverse reaction (oxidation of dihydrolipoic acid with $NADP^+$). In contrast, LipDH was only 0.048 times as efficient in the forward reaction as compared to the reverse reaction (using NADH and NAD^+). We conclude that all or part of the previously described NADPH-dependent lipoamide dehydrogenase (diaphorase) activities in mammalian systems should be attributed to TR. Our results suggest that in mammalian cells a significant part of the therapeutically important reduction of lipoic acid is catalyzed by thioredoxin reductase.

Thioredoxin reductase (TR), lipoamide dehydrogenase (LipDH) and glutathione reductase (GR) belong to a family of pyridine nucleotide-disulfide oxidoreductases containing FAD and a redox active disulfide (1). The two latter enzymes are highly similar homo-dimeric enzymes with 50 kDa subunits conserved between all species. In contrast, the properties of thioredoxin reductase (EC 1.6.4.5) differs between *Escherichia coli* and mammalian cells (2). The reaction catalyzed by TR from all species is:

$$NADPH + H^+ + Trx\text{-}S_2 \rightleftarrows NADP^+ + Trx\text{-}(SH)_2$$

where $Trx\text{-}S_2$ is oxidized thioredoxin and $Trx\text{-}(SH)_2$ reduced thioredoxin. Thioredoxin (12 kDa) in reduced form is an efficient protein disulfide reductase with numerous functions, including function as hydrogen donor of ribonucleotide reductase, regulation of chroloplast enzyme activities, redox control of transcription factors and cytokine effects on normal and malignant cells. For reviews on thioredoxin, see (3-5).

Thioredoxin reductase from *E. coli* with a subunit M_r of 35 kDa has been studied in depth, with cloning, sequencing and a high resolution X-ray structure of the enzyme (6, 7). The three-dimensional structure including the amino acid sequence around the redox active disulfide in

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Abbreviations used are: TR, thioredoxin reductase; LipDH, lipoamide dehydrogenase; GR, glutathione reductase; Trx, thioredoxin; $Trx\text{-}S_2$, oxidized Trx; $Trx\text{-}(SH)_2$, reduced Trx; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); lipoamide, DL-6,8-thioctic acid amide; lipoic acid, oxidized DL-6,8-thioctic acid; dihydrolipoic acid, reduced DL-6,8-thioctic acid; TE buffer, 50 mM Tris-Cl, 2 mM EDTA, pH 8.0.

0006-291X/96 $18.00

E. coli TR is strikingly different from the corresponding consensus structures of GR and LipDH and it was concluded that the disulfide reductase activities of *E coli* TR and GR must have evolved convergently (7). Mammalian TR was originally purified from calf liver and thymus (8), and to homogeneity from rat liver (9). The rat liver enzyme showed subunits with M_r of 58,000 and the human placenta enzyme had similar properties with even higher subunit M_r (10). Mammalian TR, in contrast to the *E. coli* enzyme, shows a broad substrate specificity and reduces not only thioredoxins from many species (8) but also several low molecular weight substrates including 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), vitamin K, selenodiglutathione, selenite, lipid hydroperoxides, and alloxan (reviewed in (4). Inhibitors of mammalian TR include antitumour quinones (11, 12), nitrosoureas (13), 13-*cis*-retinoic acid (14), and 1-chloro-2,4-dinitrobenzene (DNCB) (15). DNCB also induces NADPH oxidase activity after alkylation of mammalian TR (15), which resembles the NADPH oxidase (or diaphorase) activity of both glutathione reductase (16) and lipoamide dehydrogenase (1).

We have sequenced a large number of internal peptides of bovine TR and cloned and sequenced the rat enzyme (manuscript submitted) which largely confirms the cDNA sequence of a putative human TR recently reported by Powis and coworkers (17). The sequences revealed a close structural relationship between mammalian TR and GR or LipDH. We have therefore reinvestigated the activity of mammalian TR with the principal substrates for GR and LipDH, and found that lipoamide and lipoic acid were efficient substrates for the enzyme.

Lipoic acid bound in amide linkage to transacetylase or transsuccinylase is an essential cofactor in the mitochondrial oxoacid dehydrogenase complexes (1, 18, 19). Recently lipoic acid has gained interest as an antioxidant, with potential therapeutic applications in a range of conditions, including ischemia-reperfusion injuries, cataract formation, HIV activation, neurodegeneration and radiation injury (19).

Both lipoic acid and dihydrolipoic acid are present extracellularly and human plasma levels have been reported to be 1-25 ng/ml and 33-145 ng/ml, respectively (20). Exogenously administered lipoic acid is rapidly and to a high extent reduced to dihydrolipoic acid by mammalian cells (19, 21, 22). LipDH cannot be the sole enzyme catalyzing this reduction, since cells which lack LipDH, like erythrocytes, still reduce lipoic acid to dihydrolipoic acid by an NADPH dependent reaction (22). Glutathione reductase is an NADPH dependent lipoic acid reducing enzyme, but the efficiency in this reaction was very low compared to reduction of GSSG (22, 23).

MATERIALS AND METHODS

Material. 2′,5′-ADP-Sepharose and Q Sepharose were from Pharmacia Inc. Recombinant human Trx was prepared as described (24) while *E. coli* Trx came from IMCO (Sweden). Porcine LipDH was purchased from Sigma, as was lipoamide (oxidized DL-6,8-thioctic acid amide), lipoic acid (oxidized DL-6,8-thioctic acid) and dihydrolipoic acid (reduced DL-6,8-thioctic acid). Dihydrolipoic acid was used within two hours upon opening of the sealed ampule. All other chemicals used were of analytical grade or better. TR from bovine liver and thymus, rat liver or human placenta was purified based upon the method described earlier (9). The purifications typically resulted in 2.5 mg of TR from 1 kg of tissue, homogenous as judged by SDS PAGE and with a specific activity in DTNB reduction (8) of more than 1000 A_{412}/min/mg.

Ion exchange chromatography. Aliquots of TR (6 μg in 200 μl TE buffer) were loaded on a Mono Q PC 1.6/5 column (SMART system, Pharmacia Biotech Inc., Sweden) equilibrated with TE buffer. TE buffer was allowed to run through until UV baselines were steady (approximately 5 min at 100 μl/min) whereupon protein was eluted with a linear NaCl gradient 0 -0.3 M in TE buffer in 20 min. Fractions of 100 μl were continuously collected and stored at −20°C until analyzed for enzyme activity. Protein content in each fraction was determined using the SMART manager software for calculation of the integrated absorbance at 280 nm.

Enzyme activity determination. Enzyme activity was measured spectrophotometrically at 20°C in TE buffer as described earlier (8, 9), with the following modifications. Screening of activity in fractions of the MonoQ chromatography (Figure 2) was carried out in 96-well Cel-Cult plates (Sterilin Ltd., England) and consumption of NADPH was measured through decrease of absorbance at 340 nm determined using the THERMOmax microplate reader (Molecular

Devices Corp., CA) with the kinetic application of the accompanying SOFTmax software. Samples (10 μl) were added to wells containing 150 μl TE buffer with 200 μM NADPH and substrate, i.e. either SeO_3^{2-} (200 μM), lipoamide (400 μM) or insulin (100 μM), the latter supplemented with either human (5 μM) or *E coli* (5 μM) Trx. In each case, reference wells contained only TE buffer and 200 μM NADPH. Reduction of DTNB (500 μM) in the fractions was performed in the same manner, but in the presence of 300 μM NADPH and the reduction of DTNB determined by increase of absorbance at 405 nm (instead of 412 nm). A well containing TE buffer, 300 μM NADPH and 500 μM DTNB but no enzyme was used as reference.

When lipoamide and lipoic acid as substrates were further analyzed (Tables I and II, Figure 2), enzyme activity was determined at 20°C using a Shimadzu double-beam spectrophotometer with semi-micro cuvettes containing 500 μl TE buffer (pH 8.0) with 200 μM pyridine nucleotide, enzyme and lipoamide or lipoic acid (0-2500 μM), with everything included except lipoamide or lipoic acid in the reference cuvette. In each case, 5 μl of pure enzyme was added, which gave final TR concentrations of 1.2 nM (human placenta TR), 6.9 nM (calf thymus TR), 12.4 nM (rat liver TR) and 13.8 nM (calf liver TR), as determined in separate experiments by DTNB reduction calculating with an M_r for the holoenzyme of 116000 and assuming 1200 A_{412} units/min/mg for the pure enzyme (4). Porcine LipDH from Sigma (10.4 mg/ml) was diluted in TE buffer at the time for experiments and an M_r of 100 000 was used in calculations of concentrations (3 μl of 100 times diluted LipDH in 500 μl was considered to give 6.24 nM LipDH in final concentration). Conversion of pyridine nucleotide was determined by decrease (oxidation of NAD(P)H) or increase (reduction of $NAD(P)^+$) of absorbance at 340 nm and using a molar extinction coefficient of 6200 $M^{-1}cm^{-1}$. Lipoamide and lipoic acid were dissolved in EtOH as 50 mM stock solutions and EtOH was therefore added to an equal final concentration of 4% in all cuvettes. In each case, enzyme and pyridine nucleotide was added first, pre-incubated 5 min to reduce the enzyme, whereafter substrates were added and activity was measured.

RESULTS

At the onset of this study, we screened several of our preparations of purified mammalian TR with the primary substrates of GR and LipDH. GSSG was confirmed not to be a substrate, in agreement with previous results (9). However, we found a significant NADPH dependent

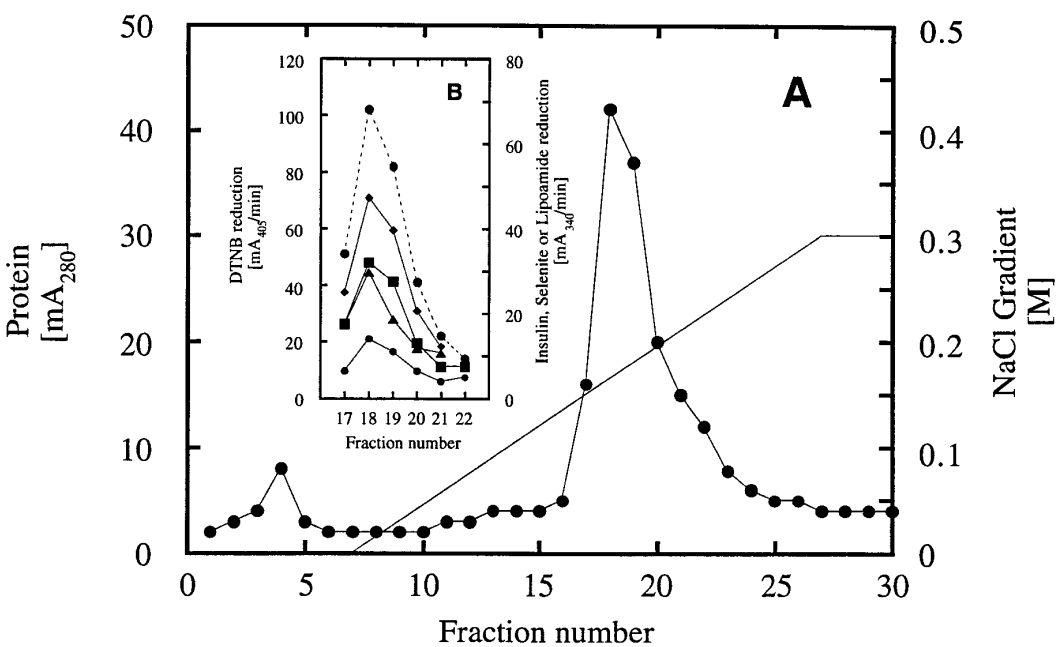

FIG. 1. Ion exchange chromatography of mammalian TR. Purified calf thymus TR was analyzed on a column of MonoQ anion exchanger with an isocratic NaCl gradient as shown in A (●; protein profile, solid line; salt gradient). B (inset) shows the enzymatic activity in those fractions containing protein, using DTNB (●, dashed line), insulin (coupled to human Trx, ♦, or *E coli* Trx, ▲), selenite (■), or lipoamide (●) as substrates.

TABLE I
Kinetic Parameters of Mammalian Thioredoxin Reductase in Reduction of Lipoamide, Reduction of Lipoic Acid, and Oxidation of Dihydrolipoic Acid[a]

Source of TR	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Calf thymus	0.49	503	1035
Calf liver	1.00	918	913
Human placenta	0.51	1102	2182
Rat liver	0.86	1296	1498
Substrate	Kinetic parameters of rat liver TR for lipoic acid and dihydrolipoic acid		
Lipoic acid	0.71	368	518
Dihydrolipoic acid[b]	0.88	173	196

[a] Consumption of pyridine nucleotide was determined by following the change of absorbance at 340 nm using a molar extinction coefficient of 6200 M^{-1} cm^{-1}. Assays were performed at 20°C,pH 8.0, in 50 mM Tris, 2 mM EDTA, and 200 μM NADPH with 3–15 nM TR (depending on preparation) and 0–2500 μM lipoamide or lipoic acid, as described in further detail in Materials and Methods. The kinetic parameters given in the table are those derived if Michaelis-Menten kinetics are fitted to the experimental data. The fitted Michaelis-Menten plots in reduction of lipamide and lipoic acid and oxidation of dihydrolipoic acid by rat liver TR are shown in Fig. 2.

[b] $NADP^+$ used instead of NADPH.

lipoamide reduction in all TR preparations that we analyzed. To exclude contamination of lipoamide dehydrogenase, one of the pure calf thymus TR preparations (showing one single distinct band on SDS gel) was further analyzed with MonoQ ion exchange chromatography. It was clear that previously known TR-catalyzed reactions (Trx-dependent insulin reduction as well as reduction of DTNB or selenite) co-eluted in one single peak together with reduction of lipoamide (Figure 1). The same result was repeated with two additional TR preparations; one from calf thymus and one from rat liver (data not shown). We also tested TR from *E. coli* for lipoamide reduction, but this enzyme did not reduce lipoamide to any detectable level (data not shown).

Kinetic parameters in the NADPH dependent lipoamide reduction by human, rat and calf TR were then determined (Table I). For the rat liver TR preparation, that had the highest specific activity, reduction of lipoic acid as well as the corresponding reverse reaction ($NADP^+$ coupled dihydrolipoic acid oxidation) was also analyzed (Table I). Also lipoic acid and dihydrolipoic acid were good substrates, with K_m values in the same range as those for lipoamide (Table I). In none of the reactions (reduction of lipoamide or lipoic acid with NADPH or oxidation of dihydrolipoic acid with $NADP^+$) could any cooperativity be observed and the experimental data were easily fitted to classic Michaeli-Menten type kinetics (Figure 2).

To compare the activities of mammalian TR with those of mammalian LipDH, both NADP(H) and NAD(H) were used as pyridine nucleotides under equivalent conditions (20°C, 50 mM Tris, 1 mM EDTA, pH 8.0, 200 μM pyridine nucleotide and 1 mM lipoic acid derivative) with both LipDH and TR. The results of these assays are given in Table II. The reduction of lipoamide was ten times more efficient by LipDH using NADH than by TR using

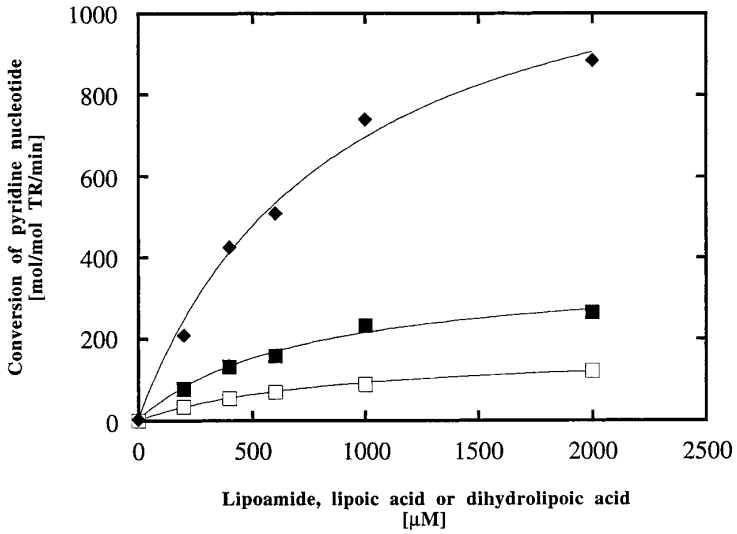

FIG. 2. Reduction of lipoamide and lipoic acid and oxidation of dihydrolipoic acid by rat liver TR. TR from rat liver (7.4 nM) was analyzed for reduction of lipoamide (♦) or lipoic acid (■) at 20°C, pH 8.0 in 50 mM Tris-Cl, 2 mM EDTA, and 200 μM NADPH or oxidation of dihydrolipoic acid (□) using 200 μM $NADP^+$ instead of NADPH. The curves fitted to the data are Michaelis-Menten plots and the derived kinetic parameters are given in Table I.

NADPH (9215 min^{-1} vs. 889 min^{-1}). TR, on the other hand, was fifteen times more efficient in reducing lipoic acid than was LipDH (297 min^{-1} vs. 20.3 min^{-1}). It was also clear that TR was more efficient in the forward reaction, i.e. reducing lipoic acid, as compared to the reverse reaction, i.e. catalyzing oxidation of dihydrolipoic acid (ratio = 2.49). The opposite, however, was true for LipDH, that was 20 times more efficient catalyzing the reverse reaction (ratio between forward and reverse reactions = 0.048).

DISCUSSION

Our results show that mammalian thioredoxin reductase efficiently catalyzes NADPH-dependent lipoamide and lipoic acid reduction. This reduction of lipoamide and lipoic acid illustrates the close relationship of the mammalian TR with other enzymes of the pyridine nucleotide disulfide oxidoreductase family, such as LipDH and GR, which has interesting implications since TR of the *E. coli* type appears not to have evolved from a common ancestral protein (7). It should be noted that we did not see evidence of cooperativity in the reduction of lipoamide or lipoic acid by TR, which is different from the pronounced cooperativity seen in the case of LipDH (1).

The efficient reduction of lipoic acid by mammalian TR adds to its known wide substrate specificity (1-4). The strong 20-fold preference of LipDH for dihydrolipoic acid oxidation compared to lipoic acid reduction under our assay conditions (Table II) can probably in part be explained by a lack of NAD^+ in the assay, since NAD^+ is a strong positive effector (1). TR readily reduced lipoic acid under without $NADP^+$, demonstrating differences in enzyme mechanism between these two enzymes. The K_m-values of mammalian TR for lipoic acid and its derivatives of 0.49 - 1.0 mM should be compared with K_m values of 2.5 - 5 μM for mammalian Trx (8, 9), illustrating the higher affinity of the enzyme for its natural substrate thioredoxin. However, the k_{cat} values of 503 - 1296 min^{-1} for lipoamide demonstrate an

TABLE II
Activity of Mammalian TR and LipDH in Reduction of Lipoamide and Lipoic Acid and in the Reverse Reaction Oxidizing Dihydrolipoic Acid, Using Either NADP or NAD as Pyridine Nucleotides

Substrate	Pyridine nucleotide	Enzyme activity (mol pyridine nucleotide/mol enzyme/min)[a]	
		TR	LipDH
Lipoamide	NADPH	889	<1
	NADH	43.4	9215
Lipoic acid	NADPH	297	<1
	NADH	<1	20.3
Dihydrolipoic acid	$NADP^+$	119	12.9
	NAD^+	23.8	424
Substrates compared		Ratio in activity between substrates compared	
Lipoamide/lipoic acid		2.99[b]	454[c]
Lipoic acid/dihydrolipoic acid		2.49[d]	0.048[e]

[a] All assays were performed at 20°C in 50 mM Tris, pH 8.0, and 2 mM EDTA with 1 mM lipoic acid derivative and 200 μM pyridine nucleotide. The concentration used of purified rat liver TR was 7.4 nM and that of porcine heart LipDH was 6.2 nM. Activity was determined as oxidation or reduction of the pyridine nucleotides by following the change in absorbance at 340 nm using a molar extinction coefficient of 6200 M^{-1} cm^{-1}.

[b] With NADPH.

[c] With NADH.

[d] Using NADPH with lipoic acid and $NADP^+$ with dihydrolipoic acid.

[e] Using NADH with lipoic acid and NAD^+ with dihydrolipoic acid.

efficient reaction, when compared to the corresponding values of 3000 - 4200 min^{-1} for different mammalian thioredoxins (9, 24).

Several NADPH-dependent diaphorases or NADPH oxidases, with varying degree of overlapping lipoamide dehydrogenase activity, have been described (25-29), many for which the true nature has not yet been fully characterized. Also, NADPH-dependent diaphorase activity has been associated with NO synthase expression in the nervous system, but additional neuronal NADPH-dependent diaphorase activity which is not dependent on NO synthase has been clearly demonstrated (30, 31). It will be important to determine how TR contributes to these less characterized diaphorase activities. It is known that the enzyme is present in the nervous system (32, 33) and the NADPH oxidase activity of TR (15) also readily reduces nitro blue tetrazolium (NBT), the classic substrate in determination of neuronal NADPH dependent diaphorase activity (Anér, Nordberg and Holmgren, unpublished observations).

It should be noted that mammalian lipoamide dehydrogenase is a mitochondrial enzyme as part of several α-keto acid dehydrogenase complexes (1, 18) whereas TR is a cytosolic enzyme with some association with membrane structures (33, 34). The association of TR with the plasma membrane is worth emphasizing, since lipoamide dehydrogenase activity has also been detected in plasma membrane fractions (35).

Dihydrolipoic acid and lipoic acid have different antioxidant properties and reduction of lipoic acid is a necessary prerequisite for several of its antioxidant effects (19). The lipoic acid reducing activity of mammalian TR should therefore play a significant role in the metabolism of this compound and for activation of its many antioxidant and redox regulatory qualities.

ACKNOWLEDGMENTS

This study was supported by the Swedish Cancer Society (961), the Swedish Medical Research Council (13X-3529), Inga-Britt and Arne Lundbergs Stiftelse, Knut and Alice Wallenbergs Stiftelse, Makarna Agnes och Gustaf Backlunds fond för cancerforskning, Stiftelsen Sigurd och Elsa Goljes minne, and the Karolinska Institute.

REFERENCES

1. Williams, C. H., Jr. (1992) *in* Chemistry and Biochemistry of Flavoenzymes. (Müller, F., Ed.) pp. 121–211, CRC Press, Boca Raton, FL.
2. Holmgren, A. (1989) *J. Biol. Chem.* **264,** 13963–13966.
3. Holmgren, A. (1985) *Annu. Rev. Biochem.* **54,** 237–271.
4. Holmgren, A., and Björnstedt, M. (1995) *Methods Enzymol.* **252,** 199–208.
5. Yodoi, J., and Tursz, T. (1991) *Adv. Cancer Res.* **57,** 381–411.
6. Waksman, G., Krishna, T. S., Williams, C. H., Jr., and Kuriyan, J. (1994) *J. Mol. Biol.* **236,** 800–816.
7. Kuriyan, J., Krishna, T. S. R., Wong, L., Guenther, B., Pahler, A., Williams, C. H., Jr., and Model, P. (1991) *Nature* **352,** 172–174.
8. Holmgren, A. (1977) *J. Biol. Chem.* **252,** 4600–4606.
9. Luthman, M., and Holmgren, A. (1982) *Biochemistry* **21,** 6628–6633.
10. Oblong, J. E., Gasdaska, P. Y., Sherrill, K., and Powis, G. (1993) *Biochemistry* **32,** 7271–7277.
11. Mau, B.-L., and Powis, G. (1992) *Biochem. Pharm.* **43,** 1613–1620.
12. Mau, B.-L., and Powis, G. (1992) *Biochem. Pharm.* **43,** 1621–1627.
13. Schallreuter, K. U., Gleason, F. K., and Wood, J. M. (1990) *Biochim. Biophys. Acta* **1054,** 14–20.
14. Schallreuter, K. U., and Wood, J. M. (1989) *Biochem. Biophys. Res. Commun.* **160,** 573–9.
15. Arnér, E. S. J., Björnstedt, M., and Holmgren, A. (1995) *J. Biol. Chem.* **270,** 3479–3482.
16. Carlberg, I., and Mannervik, B. (1980) *FEBS Lett.* **115,** 265–268.
17. Gasdaska, P. Y., Gasdaska, J. R., Cochran, S., and Powis, G. (1995) *FEBS Lett.* **373,** 5–9.
18. Perham, R. N. (1991) *Biochemistry* **30,** 8501–8512.
19. Packer, L., Witt, E. H., and Tritschler, H. J. (1995) *Free Rad. Biol. Med.* **19,** 227–250.
20. Teichert, J., and Preiss, R. (1992) *Int. J. Clin. Pharmacol. Ther. Toxicol.* **30,** 511–512.
21. Handelman, G. J., Han, D., Tritschler, H., and Packer, L. (1994) *Biochem. Pharmacol.* **47,** 1725–1730.
22. Constantinescu, A., Pick, U., Handelman, G. J., Haramaki, N., Han, D., Podda, M., Tritschler, H. J., and Packer, L. (1995) *Biochem. Pharmacol.* **50,** 253–261.
23. Pick, U., Harmaki, N., Constantinescu, A., Handelman, G. J., Tritschler, H. J., and Packer, L. (1995) *Biochem. Biophys. Res. Commun.* **206,** 724–730.
24. Ren, X., Björnstedt, M., Shen, B., Ericson, M. L., and Holmgren, A. (1993) *Biochemistry* **32,** 9701–8.
25. Cross, A. R., Yarchover, J. L., and Curnutte, J. T. (1994) *J. Biol. Chem.* **269,** 21448–21454.
26. Gasdaska, P. Y., Fisher, H., and Powis, G. (1995) *Cancer Res.* **55,** 2542–2547.
27. Carlberg, I., Sahlman, L., and Mannervik, B. (1985) *FEBS Lett.* **180,** 102–106.
28. Otsuka-Murakami, H., and Nisimoto, Y. (1995) *FEBS Lett.* **361,** 206–210.
29. Sreider, C. M., Grinblat, L., and Stoppani, A. O. M. (1990) *Biochem. Pharmacol.* **40,** 1849–1857.
30. Afework, M., Tomlinson, A., and Burnstock, G. (1994) *Cell Tissue Res.* **276,** 133–141.
31. Hope, B. T., Michael, G. J., Knigge, K. M., and Vincent, S. R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88,** 2811–2814.
32. Hansson, H.-A., Holmgren, A., Norstedt, G., and Rozell, B. (1989) *Exp. Eye Res.* **48,** 411–420.
33. Rozell, B., Hansson, H. A., Luthman, M., and Holmgren, A. (1985) *Eur. J. Cell Biol.* **38,** 79–86.
34. Schallreuter, K. U., Jänner, M., Mensing, H., Breitbart, E. W., Berger, J., and Wood, J. M. (1991) *Int. J. Cancer* **48,** 15–9.
35. Danson, M. J. (1988) *Biochem. Soc. Trans.* **16,** 87–89.