Lipoic Acid in Wheat Grains

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Lipoic acid was found to be present at low levels in wheat germ (ca. 0.1 ppm) but was not detected in flour or semolina. The reduced form of lipoic acid provided the reducing equivalents for thioredoxin in the reduction of proteins of wheat endosperm (albumins, globulins, gliadins, and glutenins). The results indicate that, while lipoic acid could function to reduce thioredoxin physiologically, its importance to technologies based on flour or semolina is questionable.

INTRODUCTION

Lipoic acid (thioctic acid) is a naturally occurring low molecular weight dithiol compound needed for the oxidation of α-keto acids in aerobic cells (Reed *et al.*, 1951; Schmidt *et al.*, 1965). Knowledge of the abundance of lipoic acid in cereals has become of increased interest as a result of the recognition of the importance of disulfide bond reduction in cystine-rich proteins of a variety of seeds (Wada and Buchanan, 1981; Johnson *et al.*, 1987; Kobrehel *et al.*, 1991, 1992; Jiao *et al.*, 1992; Shin *et al.*, 1993). The disulfide proteins of wheat endosperm were shown to be reduced *in vivo* in conjunction with their proteolytic breakdown during germination. Reduction of the major proteins was found to be specific for thioredoxin *in vitro*. Thioredoxin has also been shown to strengthen dough prepared from wheat of poor cooking quality (Wong *et al.*, 1993).

Thioredoxin is a small (12 kDa) ubiquitous protein with a catalytically active disulfide group. In heterotrophic tissues, thioredoxin is considered to be reduced by NADPH by way of NADP-thioredoxin reductase (NTR), a flavin enzyme (Florencio *et al.*, 1988; Johnson *et al.*, 1987; Suske *et al.*, 1979) (eq 1). The reduced form of lipoic acid has long been known also to be capable of reducing thioredoxin (eq 2), but the physiological importance of the reaction is unclear (Holmgren, 1985).

$$\text{NADPH} + \text{H}^+ + \text{thioredoxin } h_{ox} \xrightarrow{\text{NTR}} \text{NADP} + \text{thioredoxin } h_{red} \quad (1)$$

$$\text{lipoic acid}_{red} + \text{thioredoxin}_{ox} \rightarrow \text{lipoic acid}_{ox} + \text{thioredoxin}_{red} \quad (2)$$

Lipoic acid was reported to be present in wheat flour (endosperm) more than three decades ago (Dahle and Sullivan, 1960; Sullivan *et al.*, 1961; Morrison and Coussin, 1962; Swatditat and Tsen, 1973) and was shown to modify the properties of flour (Dahle and Hinz, 1966). The reported values for the content of lipoic acid in flour differ greatly, and the range (1-1000 ppm) is such that it cannot be readily ascribed to variety differences. Accordingly, on the basis of published studies, it is difficult to assess whether lipoic acid could play a significant technological role in reducing thioredoxin in grain preparations.

As a result of this gap, lipoic acid has been analyzed in wheat by taking advantage of recently developed analytical techniques. The effect of lipoic acid-reduced thioredoxin on wheat proteins has also been determined.

MATERIALS AND METHODS

Plant Materials. Seeds of durum (*Triticum durum* Desf. cv. Primadur) and bread wheat (*Triticum aestivum* cv. Archamp and cv. Mission) were milled in a pilot mill.

Reagents and Fine Chemicals. Oxidized and reduced lipoic acid, NADPH, and monobromobimane (mBBr) were obtained from Sigma Chemical Co. (St. Louis, MO); acrylamide and bis-(acrylamide) were from BDH (BDH Ltd., Poole, England). TEMED was obtained from Kodak Inc. (Rochester, NY) and ammonium persulfate from Merck Chemical Co. (Darmstadt, Germany). Other chemicals were of the highest quality available and were obtained from commercial sources.

Enzymes and Proteins. *Escherichia coli* thioredoxin and NTR were isolated from cells transformed to overexpress each protein. The thioredoxin strain containing the recombinant plasmid pFPI and the NTR strain containing the recombinant plasmid pPMR21 were kindly provided, respectively, by Dr. J.-P. Jacquot (de Lamotte-Guéry *et al.*, 1991) and Drs. M. Russel and P. Model (Russel and Model, 1988). The isolation procedures were as described in those studies with the following changes: cells were broken in a Ribi cell fractionator at 25 000 psi, and NTR was purified as described by Florencio *et al.* (1988) without the red agarose step. NADP-malate dehydrogenase (EC 1.1.1.82) was purified from corn leaves according to the method of Jacquot *et al.* (1981).

Isolation of Lipoic Acid from Wheat. Lipoic acid was isolated from several wheat products: ground whole wheat, germ, semolina, flour, and protein fractions. Protein fractions were obtained from the wheat products by extracting first with 5 mL·g^{-1} deionized water; sodium myristate (80 mg·g^{-1}) was then added, followed by additional water (5 mL·g^{-1}). Lipoic acid extractions were carried out with or without prior acid hydrolysis of the raw material. Protein extracts were lyophilized prior to extraction.

The method for acid hydrolysis was adapted from that of Morrison and Coussin (1962). Hydrolysis was performed in duplicate with 30 g of the wheat material in question and 200 mL of 6 N HCl by boiling for 2 h with constant nitrogen purging. After hydrolysis, samples were extracted with methylene chloride; the resultant organic fraction was evaporated to dryness under vacuum and stored at 4 °C under nitrogen.

When hydrolysis was omitted, 10 g of material was extracted with 50 mL of methylene chloride by using the Soxtec system

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0021-8561/94/1442-1110$04.50/0

HT 1043 extraction unit from Tecator (Höganäs, Sweden). The material was immersed for 1 h in methylene chloride and rinsed for 2 h at 45 °C with the same solvent. The extracts were evaporated to dryness under vacuum and stored at 4 °C.

Thin-Layer Chromatography. The procedure of Swatditat and Tsen (1973) was followed. Wheat extracts were spotted on glass plates (20 × 20 cm, coated with a 0.5-mm layer of silica gel G); oxidized lipoic acid served as a standard. The plates were developed ascendingly in a saturated chamber sequentially with three different solvent systems: (i) ethyl ether/petroleum ether (bp 35–65 °C)/acetic acid (60:40:2 v/v/v); (ii) hexane (100); (iii) water-saturated butanol:acetone (50:50 v/v). After development, the plates were air-dried and stained with iodine gas. For further characterization, spots with R_f values corresponding to those of known lipoic acid were collected and eluted sequentially with methylene chloride and methyl alcohol. The eluates were pooled, evaporated to dryness, and stored at 4 °C under nitrogen.

Covalent Affinity Chromatography. A chromatography method specific for dithiol compounds was performed according to that of Pratt *et al.* (1989) except that benzene was replaced by methylene chloride.

Capillary Gas Chromatography. The method of Paquot and Hautfenne (1987) was followed. Total extractable lipids containing lipoic acid were hydrolyzed with 0.5 M NaOH in methanol at boiling temperature for 10 min, methylated with boron trifluoride–methanol, and extracted with hexane. The preparation was then fractionated on a Delsi gas chromatograph (Model DI 700) equipped with a FID and a Shimadzu integrator (Model C-R4A). The chromatograph was fitted with a DB1 (J&W) 30-m fused silica capillary column of 0.25-mm i.d., cross-linked with methyl silicone, film thickness 0.2 μm. The column was developed at 140 °C for 20 min, temperature was increased (7 °C/min) to 280 °C and then maintained for 15 min. Both injector and detector temperatures were 280 °C. Hydrogen was used as a carrier gas with a head pressure of 10 psi. The reduced and oxidized lipoic acids were used as standards for the identification of lipoic acid.

Extraction of Wheat Proteins. The sequential extraction of wheat proteins, yielding three fractions, albumins–globulins, gliadins, and glutenins, was carried out as described earlier (Kobrehel *et al.*, 1992).

Reduction, mBBr Fluorescent Labeling, and SDS–Polyacrylamide Gel Electrophoresis Analysis. The method of Crawford *et al.* (1989), modified by Kobrehel *et al.* (1991), was used in this study. The reduction of target proteins, amounts as indicated, was carried out in 70 mM Tris-HCl buffer, pH 7.9, in a final volume of 100 μL with the following reducing agents: (i) 0.625 mM reduced lipoic acid; (ii) 0.5 μM thioredoxin and 0.625 mM reduced lipoic acid; (iii) 1.25 mM NADPH, 0.07 μM NTR, and 0.5 μM thioredoxin; (iv) 10 mM neutralized reduced glutathione; (v) 10 mM 2-mercaptoethanol. For complete reduction (vi), samples were heated in 1% sodium dodecyl sulfate (SDS) and 5 mM dithiothreithol (DTT) for 3 min. After a 30-min incubation, 80 nmol of mBBr was added, and the reaction was continued for another 20 min. To stop the reaction and derivatize the excess mBBr, 10 μL of 10% SDS and 10 μL of 100 mM 2-mercaptoethanol were added. Then the samples were applied to the gels (4% stacking, 17.5% separating). SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) was carried out for 16 h at a constant current, 7 mA. The removal of excess mBBr from the gels, fluorescence photography, and protein staining and destaining were performed as described by Kobrehel *et al.* (1992). For quantitating reduction of endosperm proteins, the Polaroid negatives of fluorescent gels and dry protein stained gels were scanned with a laser densitometer (Pharmacia-LKB Ultroscan XL). In each case, the bands were quantified by evaluating areas of the bands with Gelscan XL software. The ratio of fluorescence to protein was used to represent the extent of protein reduction.

RESULTS

Lipoic Acid Content of Wheat. Analyses for lipoic acid were carried out on wheat germ, ground whole wheat, semolina (durum wheat), flour (bread wheat), and protein extracts. Preparations were subjected to thin-layer chromatography, and spots having an R_f value identical to that of authentic lipoic acid (Dahle and Hinz, 1966; Swatditat and Tsen, 1973) were recovered for each sample. Investigators in the past have assumed that such spots contain mainly or only lipoic acid. In our case, components in spots on the plates corresponding to lipoic acid were further purified by gas chromatography. We also extracted lipid from wheat germ and subjected this fraction to affinity and gas chromatography purification steps.

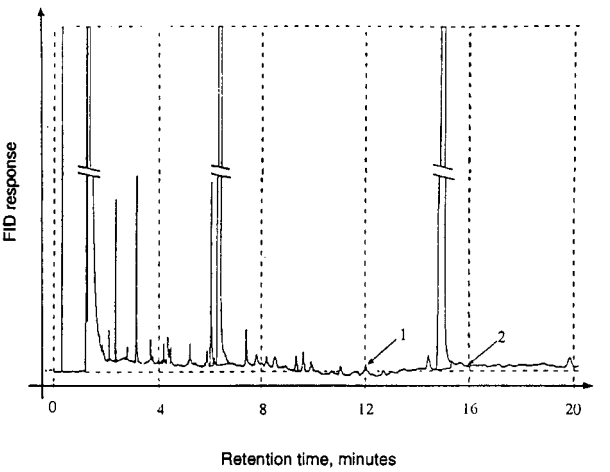

Figure 1. Capillary gas chromatograph of a wheat germ preparation. The fraction obtained from the affinity chromatography step was fractionated on a Delsi gas chromatograph (Model DI 700). The instrument was equipped with a FID and Shimadzu integrator (Model C-R4A) and fitted with a DB1 (J&W) 30-m fused silica capillary column of 0.25 mm (i.d.) cross-linked with methyl silicone (film thickness 0.2 μm). Conditions: 140 °C for 20 min, 7 °C/min to 280 °C and then 280 °C for 15 min; injector and detector both at 280 °C; hydrogen as gas carrier; head pressure, 10 psi. 1, Reduced lipoic acid; 2, oxidized lipoic acid. The other peaks were not identified.

When the original chromatography spot was subjected to these procedures, we found that the spot consisted of multiple compounds irrespective of source of material—i.e., wheat endosperm (flour or semolina), germ, or extracted proteins. Figure 1 shows the profile of a capillary gas chromatography analysis obtained with a wheat germ preparation previously purified by covalent affinity chromatography (Pratt *et al.*, 1989). A well-defined minor peak corresponding to the retention time of the reduced form of lipoic acid (peak 1 in Figure 1) was observed reproducibly in germ preparations. The peak was consistently small and of the size seen in Figure 1 for all samples examined. When co-injected with the sample, authentic lipoic acid was recovered in a peak coincident with that assumed to be lipoic acid. The oxidized form of lipoic acid (position indicated by arrow 2 in Figure 1) was not detected by this procedure because of reduction of the sample prior to chromatographic analysis.

The amounts of lipoic acid found in the germ samples were too low to allow further characterization by coupling gas chromatography to mass spectrophotometric analysis. Nonetheless, the low values seem to be real and not due to faulty methodology. Addition of 20 mg of oxidized lipoic acid to samples before extraction with methylene chloride or after hydrolysis with 1 N HCl revealed a recovery of 40% following gas chromatography, consistent with reported results (Kozma-Kovács *et al.*, 1991). Assuming this same 60% loss in the extraction of flour samples, our results indicate that wheat germ has a maximum lipoic acid content of 0.1 ppm.

In contrast to germ, we could not detect lipoic acid in endosperm preparations (flour or semolina) despite re-

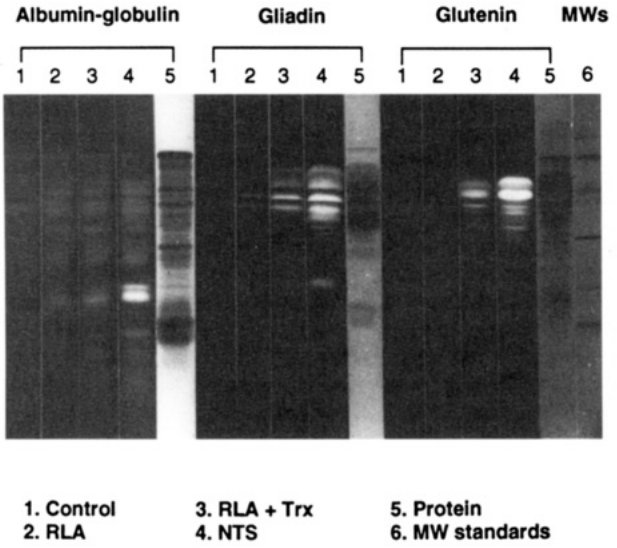

Figure 2. Reduction of wheat protein fractions with lipoic acid and thioredoxin. Treatments: 1, control; 2, reduced lipoic acid (0.625 mM); 3, reduced lipoic acid (0.625 mM) plus *E. coli* thioredoxin (0.5 μM); 4, NADPH (1.25 mM), *E. coli* thioredoxin (0.5 μM), and NTR (0.07 μM). The indicated proteins were used in the following amounts: albumin–globulin, 180 μg; gliadin, 70 μg; glutenin, 210 μg. Molecular weight standards: phosphorylase *b*, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; Kunitz soybean trypsin inhibitor, 20 000; α-lactalbumin, 14 400.

peated attempts. Several preparations were tested by capillary gas chromatography following extraction with methylene chloride and methylation with trifluoride–methanol: (1) untreated flour and semolina; (2) acid-hydrolyzed flour and semolina; (3) delipidated acid-hydrolyzed flour and semolina; (4) thin-layer chromatography fractions obtained from delipidated acid-hydrolyzed flour and semolina. The detection limit by our procedure was 20 ng of lipoic acid. On the basis of our findings, flour and semolina would contain no more than 0.03 ppm of lipoic acid.

Effectiveness of Lipoic Acid as a Reductant. In spite of evidence that lipoic acid is not a major component of wheat endosperm, its ability to reduce endosperm proteins is a relevant question, especially in view of the ability of thioredoxin to serve as a specific reductant for many of these proteins (Kobrehel *et al.*, 1992). The redox state of the major protein fractions of wheat (albumins–globulins, gliadins, glutenins) was, therefore, monitored by following reduction with lipoic acid added in either the absence or presence of thioredoxin by using the mBBr/SDS–polyacrylamide gel electrophoresis procedure. In this procedure, proteins with SH groups appear as fluorescent bands in photographs.

The results show that lipoic acid reduced albumins-globulins and a limited number of gliadins but was less effective with glutenins (Figures 2 and 3). The albumin–globulin fraction, containing soluble enzymes, was the most sensitive to reduction by lipoic acid (Figures 2 and 3) perhaps as a result of endogenous thioredoxin (Johnson *et al.*, 1987; Suske *et al.*, 1979). Thioredoxin added in the presence of lipoic acid greatly increased reduction both in the number of proteins and in their extent of reduction, although reduction was considerably less extensive than when thioredoxin was reduced with NADPH via NTR (Figures 2 and 3).

In agreement with the earlier results of Kobrehel *et al.* (1992), reduced glutathione was much less effective as a reductant than thioredoxin (Figure 4). For each protein fraction, reduction was consistently higher with thioredoxin, which could be reduced either by lipoic acid or by the NADPH and NTR components of the NADP/thioredoxin system. In the presence of thioredoxin, lipoic acid was less effective than NADPH/NTR. The extents of reduction by thioredoxin reduced via lipoic acid vs NADPH/NTR were as follows: 20% vs 40% with albu-

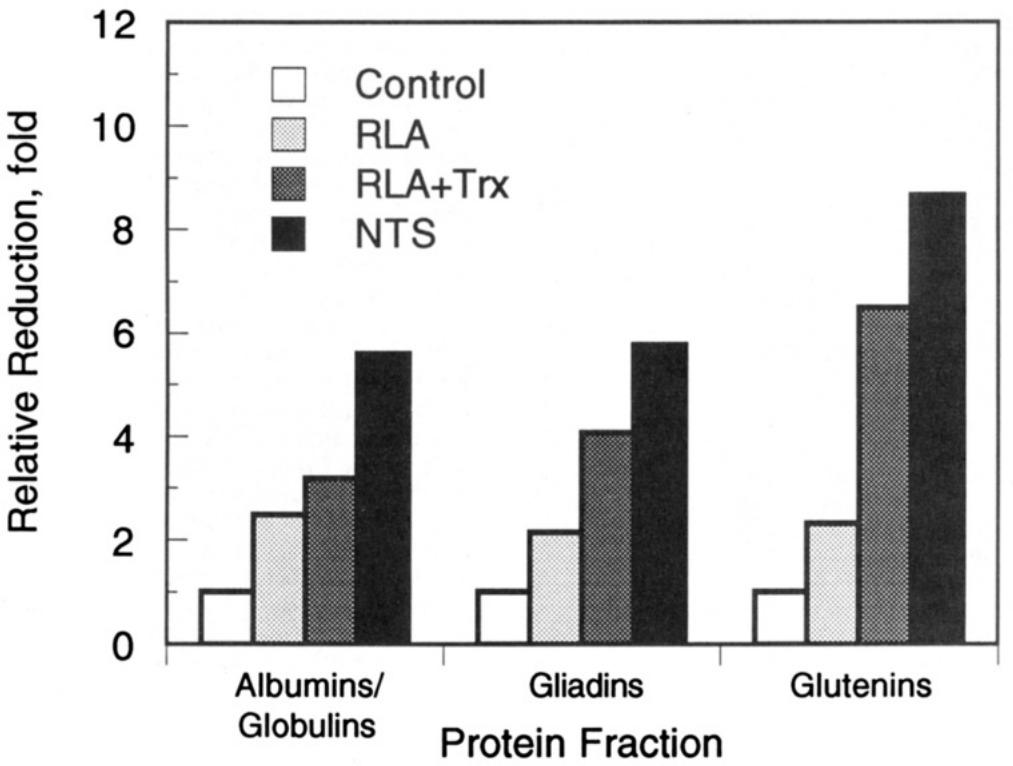

Figure 3. Reduction of wheat protein fractions with lipoic acid and thioredoxin. The negative of Figure 2 was scanned with a laser densitometer as described by Kobrehel *et al.* (1992). Trx, thioredoxin; NTS, NADP/thioredoxin system; RLA, Reduced lipoic acid.

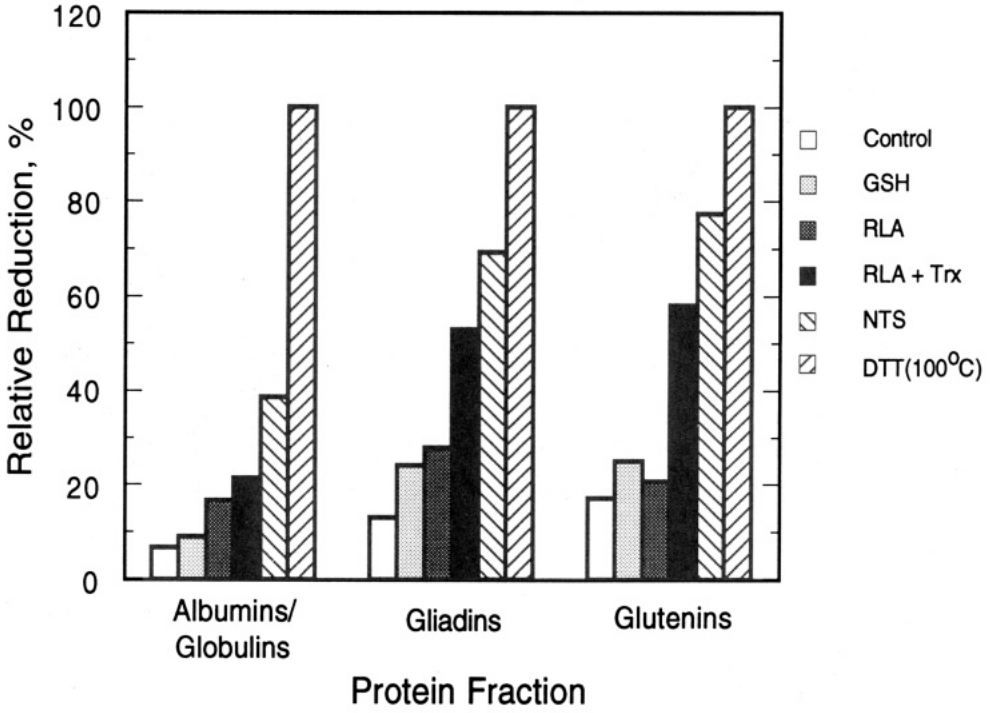

Figure 4. Reduction of wheat protein fractions by different reductants.

mins–globulins; 55% vs 70% with gliadins; and 60% vs 80% with glutenins. 2-Mercaptoethanol was without appreciable effect (data not shown).

Lipoic acid was also found to be an effective reductant of wheat proteins when monitored by the activation of chloroplast NADP–malate dehydrogenase (NADP–MDH) in the presence of thioredoxin and CM-16, a wheat α-amylase inhibitor protein (Kobrehel *et al.*, 1991) (eq 3).

$$\text{reduced lipoic acid} \rightarrow \text{thioredoxin} \rightarrow \text{CM-16} \rightarrow \text{NADP–MDH} \quad (3)$$

With CM-16 alone, lipoic acid effected a low activation of NADP–malate dehydrogenase (0.03 nkat). In the presence of a low level of *E. coli* thioredoxin (that had little effect by itself), activation was increased more than 6-fold (0.20 nkat). Similar results were obtained in an earlier study in which thioredoxin was reduced with dithiothreitol (Kobrehel *et al.*, 1991).

The above results demonstrate that lipoic acid is effective in the thioredoxin-linked reduction of proteins of the wheat endosperm *in vitro*. Lipoic acid was, however, consistently less effective than the NADP/thioredoxin system in all cases examined.

Concluding Remarks. The above results confirm the presence of lipoic acid in wheat germ, although at levels lower than earlier reported. The improved methods applied in the current analyses can account for these differences. Because of its low abundance in flour and semolina, the present results raise the question of the importance of lipoic acid to technological processes such as the making of dough. On the other hand, the results indicate that lipoic acid could act to reduce thioredoxin physiologically given the appropriate conditions.

ABBREVIATIONS USED

DTT, dithiothreitol; mBBr, monobromobimane; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NADP–MDH, NADP–malate dehydrogenase; NTR, NADP–thioredoxin reductase; NTS, NADP–thioredoxin-system; OAA, oxaloacetic acid; RLA, reduced lipoic acid; SDS, sodium dodecyl sulfate; TEMED, *N,N,N′,N′*-tetraethylethylenediamine; Tris, tris[hydroxymethyl]aminomethane.

ACKNOWLEDGMENT

We are grateful to Professor K. J. Stevenson, University of Calgary, Canada, for helpful advice on performing dithiol-specific affinity chromatography analyses. We thank Ms. Boihon Yee for the samples of thioredoxin and NTR. This research was supported in part by Grant MCB 93-16496 from the National Science Foundation.

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Received for review September 7, 1993. Revised manuscript received January 19, 1994. Accepted February 28, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1994.