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Elucidation of Antioxidant Activity of α-Lipoic Acid toward Hydroxyl Radical

Seiichi Matsugo[1*], Liang-Jun Yan[1], Derick Han[1], Hans J. Trischler[2] and Lester Packer[1+]

[1]Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA 94720-3200

[2]ASTA-Medica, D-60314 Frankfurt am Main, Germany

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Abstract: The photosensitive organic hydroperoxide, NP-III, which produces hydroxyl radicals on illumination by UVA light, was used to examine the antioxidant activity of α-lipoic acid and its derivatives toward hydroxyl radical. Apolipoprotein (apo-B) of human low density lipoprotein (LDL) and bovine serum alubumin (BSA) were irradiated with UVA in the presence of NP-III and α-lipoic acid. The oxidation of BSA and the apo-B protein of LDL by NP-III was completely suppressed by α-lipoic acid. ESR studies using dimethylpyrroline oxide (DMPO) as a spin trapping reagent also revealed that in the presence of α-lipoic acid, the DMPO-OH adduct produced from the irradiation of NP-III and DMPO completely disappeared. DMPO-OH quenching experiments were performed in the presence or absence of desferoxamine but no change in the signal intensity was found. Hence, the quenching activity of α-lipoic acid is not due to its chelating activity toward transition metals (ferrous ions). The results lead us to conclude that α-lipoic acid is an efficient hydroxyl radical quencher owing to the disulfide bond in the dithiolane ring.

The role of α-lipoic acid in energy metabolism is well known. As lipoamide, it functions as a cofactor in multienzyme complexes that catalyze the oxidative decarboxylation of α-keto acids such as pyruvate, α-ketoglutarate and branched chain α-keto acids [1]. Recently, a great deal of attention has been paid to the antioxidant activity of α-lipoic acid and its reduced form, dihydrolipoic acid [2-5] (Fig. 1). The evaluation of the antioxidant activity of α-lipoic acid toward hydroxyl radical has been reported using the metal-hydrogen peroxide system to generate hydroxyl radical [6,7]. In these studies, it is difficult to distinguish between the chelating activity of α-lipoic acid and its direct hydroxyl radical scavenging.

We have developed a novel compound that generates hydroxyl radicals upon irradiation with long wavelength UV [8], and have examined the hydroxyl radical-induced oxidative damage of DNA [9,10], cells [11, 12], and apolipoprotein [13]. Based on these findings, the antioxidant activity of α-lipoic acid toward hydroxyl radical could be examined by measuring its effect on the

* On leave from Department of Chemical & Biochemical Engineering, Faculty of Engineering, Toyama University, Gofuku 3190, Toyama 930, Japan.

+To whom correspondence should be addressed. Fax: 510-642-8313.

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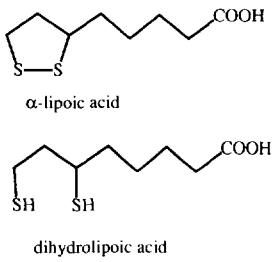


Fig. 1. Structure of α-Lipoic Acid and Dihydrolipoic Acid.

change of the ESR spectrum of the spin-trapped adduct produced from the reaction of NP III (N,N'-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetracarboxylic diimide) and DMPO (5,5-dimethyl-1-pyrroline-N-oxide) and the production of carbonylated protein from the reaction of NP-III with BSA (bovine serum alubumin) and human LDL (low density of lipoprotein).

Materials and Methods

All reagents used were of reagent grade and used without purification except where indicated. The preparation of NP-III and LDL were the same as described before [9]. α-Lipoic acid was a kind gift from ASTA Medica (Fankfurt). Dimethylpyrroline oxide was used after filtration through charcoal. ESR spectra were measured using an IBM ER 200 D-SRC electron spin resonance spectrometer with samples in a capillary tube at room temperature. UV spectra were measured using Shimadzu UV 160 U spectrophotometer and irradiation was carried out using an Oriel Corporation 68820 instrument with UVB and UVC cutoff filters.

ESR Spin Trapping Technique. Hydroxyl radical generation in the aqueous solution of NP-III was measured by the ESR spin trapping technique using the cyclic nitrone spin trap DMPO. A mixture containing 50 μl of 100 mM DMPO , 10 μl of 100 μM NP-III, 20 μl of water and defined concentration of 20 μl of α-lipoic acid was irradiated by the Oriel Corporation 68820 instrument with UVB and UVC cutoff filters for 1 min. The solution (50 μl) was transfered to a capillary tube and the capillary tube was sealed. The sealed capillary tube was placed in a cavity of the ESR spectrometer. Recordings of the spectra were made at modulation amplitude 1.25 G, frequency 100 kHz, scan range 100 G, central field 3480 G, microwave power 9.9 mW, time constant 500 ms, sweep time 200 /s, gain 1 x 10^4.

The time-dependent formation of hydroxyl radical from the NP-III was performed under similar conditions. The sample solution containing 50 μl of 100 mM DMPO, 10 μl of 100 μM of NP-III, and 40 μl of double-distilled water was irradiated from the Oriel Corporation 68820 instrument with UVB and UVC cutoff filters for defined periods. The sample solution (50 μl) was transfered to a capillary tube and measured under the conditions described above.

Oxidation of Salicylic Acid by NP-III in the Presence of α-Lipoic Acid. In this and in other irradiated NP-III solutions were prepared in acetonitrile to a concentration ten-fold the final sample concentration. Then, to a solution of 250 μl of 2 mM of salicylic acid, 50 μl of 1mM NP-III and 150 μl of 27 mM citrate and 30 mM acetate buffer was added 50ul of α-lipoic acid to final concentrations of 0,100,250, 500, 1000, 2500 and 5000 μM. Samples were then directly irradiated. A duplicate set of samples covered with foil served as dark controls. Irradiation was provided by an Oriel Corporation 68820 apparatus using UVB and UVC cutoff filters (cutoff wavelength, 320 nm) at a distance of 10 cm. Samples were irradiated for 30 min. After the reaction, the reaction mixture was subjected to HPLC-ECD system to detect the formation of 2,3-dihydroxy-benzoic acid and 2,5-dihydroxy-benzoic acid. The yield for the formation of hydroxylated salicylate was determined by comparing the peak areas to those of standards [13].

Oxidation of Bovine Serum Albumin by NP-III in the Presence of α-Lipoic Acid. The concentration of BSA was adjusted to 2 mg/ml in 0.1M sodium phosphate buffer (pH 7.2). NP-III solution of 100 μM was prepared by diluting 1 mM NP-III solution in acetonitrile by re-

distilled water. Solutions containing 800 µl of BSA, 100 µM of 100 µl of NP-III and 100 µl of α-lipoic acid at final concentrations of α-lipoic acid of 5000, 2500, 1000, 500, 250 uM were directly irradiated. A duplicate set of samples covered with foil served as dark controls. The carbonyl content of the BSA was measured as described [14].

LDL Oxidation by NP-III in the Presence of α-Lipoic Acid. Human LDL oxidation by NP-III at 10 µM concentration in the presence of defined concentration of α-lipoic acid was carried out under the same reaction conditions as those for BSA reaction. 800 µl of LDL solution was added to 100 µl of 100 µM of NP-III and 100 µl of α-lipoic acid at 50, 25, 10, 5, 2.5 1 mM concentrations. The protein concentration of LDL was 1 mg/ml, confirmed by the Lowry method [15]. The irradiation was carried out in the same manner as with BSA. After the reaction, the sample solution was treated with the method described [16] and the carbonyl content of protein of LDL was determined spectroscopically.

Results

When NP-III was irradiated with UVA light (> 320 nm) in the presence of dimethylpyrolline oxide (DMPO), the signal corresponding to the adduct (DMPO-OH) of hydroxyl radical with DMPO was observed. The formation of DMPO-OH from the reaction of 10 µM of NP-III and 50 mM DMPO reaches maximum at ca. 60 sec. irradiation and the intensity gradually decreases as the irradition time becomes longer (data not shown). The addition of α-lipoic acid to the sample decreases the formation of spin adduct significantly (Fig. 2). For example, when 10 µM of NP-III was irradiated with UVA light in the presence of 1mM of α-lipoic acid, no signal corresponding to DMPO-OH adduct was observed (third line). By decreasing the concentration of α-lipoic acid (fourth line to eighth line), the intensity of the signal corresponding to DMPO-OH was increased gradually. Without photoirradiation (first line), no signal corresponding to DMPO-OH was observed at all.

In order to confirm the possibility of transition metal effects due to iron on the spin-trapping, the experiments were performed in the presence of desferoxamine. There was no different results obtained in the presence or absence of desferoxamine (data not shown).

Hydroxyl radical quenching activity of α-lipoic acid was confirmed by the inhibition of the formation of dihydroxy-benzoic acid from the reaction of salicylic acid and NP-III. We have already reported that reaction of NP-III with salicylic acid caused the formation of 2,3- and 2,5-dihydroxybenzoic acid[14]. The formation of dihydroxybenzoic acid was completely suppressed in the presence of 5 mM of α-lipoic acid (Fig. 3). The quenching activity of α-lipoic acid was evident up to a concentration of α-lipoic acid of 500 µM concentration (five times that of NP-III).

When BSA solution was irradiated in the presence of 10 µM of NP-III for 30 min., the carbonylation of BSA increased from 2.8 nmol/mg to 44.3 nmol/mg protein. The protein carbonyl concentration of BSA was significantly decreased in the presence of α-lipoic acid. For example, in the presence of 1 mM α-lipoic acid (100 times the concentration of NP-III), the protein carbonyl concentration decreased to 14.2 nmol/mg protein under the same reaction conditions. The inhibitory effect of α-lipoic acid was observed at 250 µM of lipoic acid concentration (Fig. 4). The carbonylated protein formation was time-dependent (Fig.5).

LDL oxidation by 10 µM NP-III was examined in the presence or absence of α-lipoic acid. In the absence of α-lipoic acid, the carbonylated apolipoprotein from LDL increased to 16.76 nmol/mg protein, however, in the presence of 500 µM α-lipoic acid (50 times the concentration of NP-III), the value was lowered to 10.31 and in the presence of 5 mM of α-lipoic acid (500 times

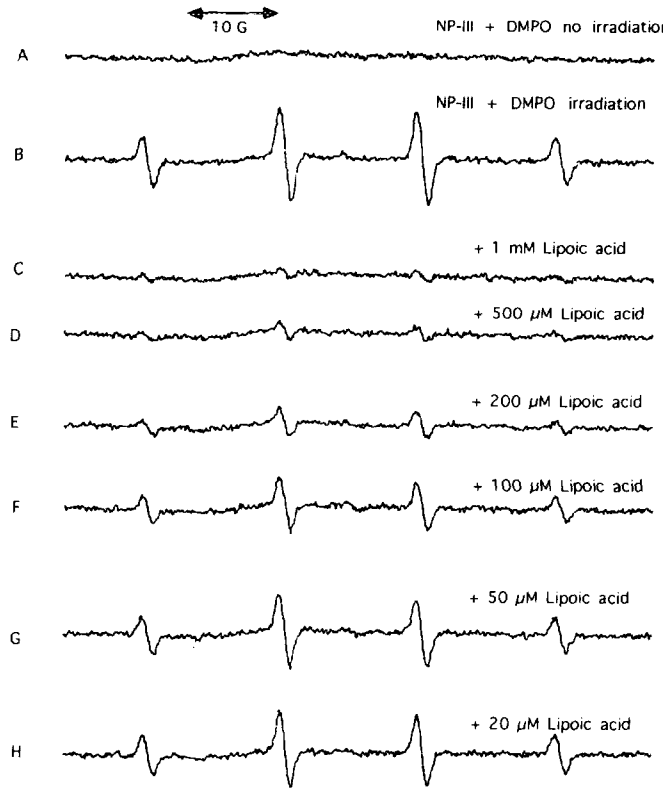
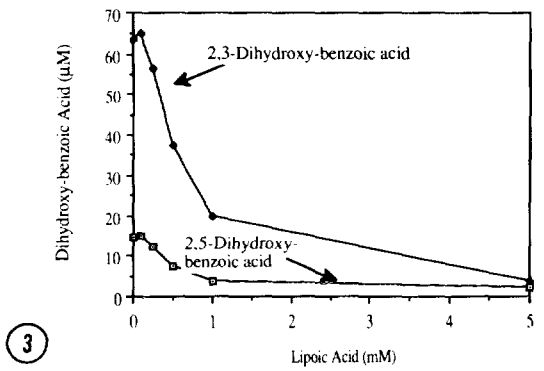


Fig. 2. ESR spin trapping studies using dimethylpyrroline oxide. DMPO (100 mM) in 0.1 M phosphate buffer (pH 7.0) was incubated with 10 μM of NP-III in the presence of various concentrations of α-lipoic acid. After 1 min. photoreaction, an aliquot (50 μl) of the reaction mixture was moved into a capillary, which was then sealed to measure the formation of DMPO-OH adduct. A, DMPO and NP-III without irradiation; B, DMPO and NP-III irradiation for 1min; C, DMPO, NP-III and α-lipoic acid (1mM) irradiation for 1 min; D, DMPO, NP-III, and α-lipoic acid (500 μM) irradiation for 1 min; E, DMPO, NP-III, and α-lipoic acid (200 μM) irradiation for 1 min; F, DMPO, NP-III, and α-lipoic acid (100 μM) irradiation for 1 min; G, DMPO, NP-III, and α-lipoic acid (50 μM) irradiation for 1 min; H, DMPO, NP-III, and α-lipoic acid (20 μM) irradiation for 1 min.

the concentration of NP-III), the formation of protein carbonyl was almost completely quenched (Fig. 6). These data are consistent with those obtained by ESR (Fig. 2).

Discussion

NP-III is a new and novel compound that is useful in studying oxidation reactions initiated by hydroxyl radicals. It's main attraction is the precision with which it can be used. It generates exclusively hydroxyl radicals and hydroxyl radical generation can be precisely controlled through controlling intensity of UVA irradiation. The generation of hydroxyl radical from NP-III was confirmed by detection of the formation of DMPO-OH adduct in the ESR spectrum. The



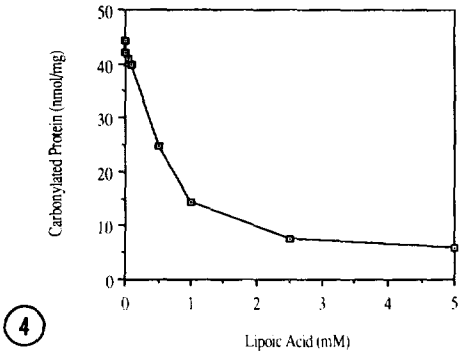


Fig. 3. Oxidation of Salicylic Acid by NP-III in the Presence of α-Lipoic Acid. Salicylic acid (2 mM) dissolved in 27 mM citrate and 30 mM acetate buffer was incubated with 1 mM of NP-III in the presence of various concentrations of α-lipoic acid. After irradiation the sample solutions were subjected to HPLC-ECD and the amounts of the hydroxylated salicylic acid were determined by comparing the peak area with those of standards of 2,3- and 2,5-dihydroxy-benzoic acid.

Fig. 4. Oxidation of Bovine Serum Albumin by NP-III in the Presence of α-Lipoic Acid. BSA (2mg / ml) was incubated with 10 μM of NP-III in the presence of various concentrations of α-lipoic acid in 0.1 M phosphate buffer solution (pH 7.0). After the irradiation, the sample solution was reacted with dinitrophenylhydrazine for 1h at room temperature then 10% trichloroacetic acid (final concentration) was added to precipitate protein. The protein pellet was washed three times with ethyl alcohol-ethyl acetate (v / v, 1 : 1) and then it was dissolved in 6 M guanidine - HCl solution. The readings were taken at 360 nm using 22000 $M^{-1}cm^{-1}$ of DNPH to calculate protein carbonyls.

formation of the DMPO-OH adduct signal was decreased by the addition of α-lipoic acid in a concentration dependent manner. From the decay curve of the ESR signal intensity of DMPO-OH, we can evaluate the rate constant of α-lipoic acid reaction with hydroxyl radical by the following equations.

(eq. 1)

$$A = [\text{DMPO-OH}]_{\text{observ.}} = k_{\text{DMPO-OH}}\,[\text{DMPO}][\cdot\text{OH}]$$

(eq.2)

$$A_0 = [\text{DMPO-OH}]_{\text{init.}} = k_{\text{DMPO-OH}}\,[\text{DMPO}][\cdot\text{OH}] + k_{\alpha\text{-lipoic acid}}\,[\alpha\text{-lipoic acid}]\,[\cdot\text{OH}]$$

(eq. 3)

$$A_0 / A = 1 + k_{\alpha\text{-lipoic acid}}\,[\alpha\text{-lipoic acid}] / k_{\text{DMPO-OH}}\,[\text{DMPO}]$$

By plotting the concentration of α-lipoic acid as X-axis and the relative intensity of A_0 / A = [DMPO] init. / [DMPO]observ. as Y-axis, a straight line can be drawn (Fig. 7). Where the slope of this line is $k_{\alpha\text{-lipoic acid}} / k_{\text{DMPO-OH}}$ [DMPO]. Introducing $k_{\text{DMPO-OH}}$ as 3.4×10^9

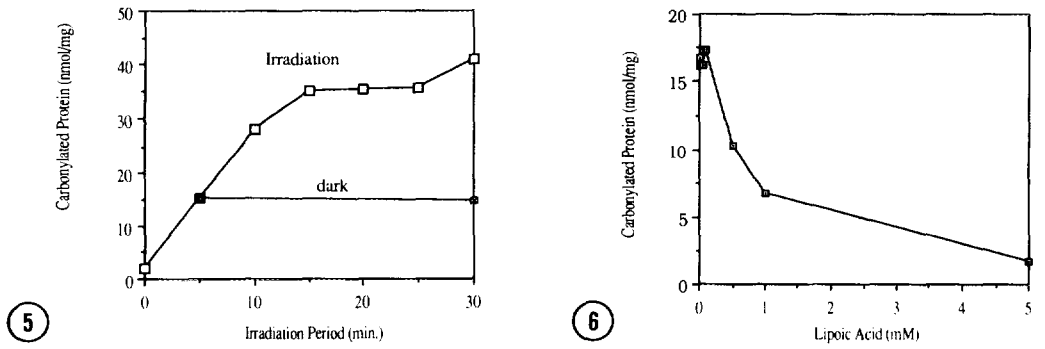


Fig. 5. Time-dependent Formation of Protein Carbonyls of BSA by the Irradiation of 10 μM of NP-III in the Presence of 500 μM of α-lipoic acid. BSA (2mg / ml) was incubated with 10 μM of NP-III in the presence of 500 μM of α-lipoic acid in 0.1 M phosphate buffer (pH 7.0). The photochemical reaction was carried out for the indicated times. After the photochemical reaction, the sample solution was reacted with 2,4-dinitrophenylhydrazine and the precipitate produced was washed three times using ethyl alcohol and ethyl acetate (v / v, 1 : 1). The carbonyl concentration of BSA was determined at 360 nm as described in the legend of Fig. 4.

Fig. 6. LDL Oxidation by NP-III in the Presence of α-Lipoic Acid. Human LDL (1.0 mg protein / ml) was incubated with 10 μM of NP-III in the presence of various concentrations of α-lipoic acid in saline solution (pH 7.4). After the photoreaction, the sample solution (1 ml) was reacted with 200 μl of dinitrophenylhydrazine (10 mM) for 1h, to which was added the solutions of 3 ml of n-hexane, 3 ml of ethyl alcohol and 500 μl of 20% SDS. The mixture was vortexed for 1 min. which was then centrifuged for 5 min. The precipitate obtained during these procedures was washed three times with ethanol : ethyl acetate (v / v, 1 : 1), then it was dissolved in 6 M urea - 6 % SDS solution (pH 6.8). The readings were taken at 360 nm using 22000 $M^{-1}cm^{-1}$ of DNPH to calculate protein carbonyls.

M^{-1} s^{-1}[17], the k α-lipoic acid is obtained as 1.92 ± 0.2 X 10^{10} $M^{-1}s^{-1}$. This value is comparable to the value of 4.70 X 10^{10} $M^{-1}s^{-1}$ obtained from the quenching of hydroxyl radical by α-lipoic acid in the deoxyribose degradation assay reported by Scott *et. al.*[7].

In the previous papers dealing with the quenching activity of α-lipoic acid toward hydroxyl radical, the quenching activity of α-lipoic acid was considered to be due to the chelating ability of α-lipoic acid but without definitive proof. To test this point the DMPO-OH signal quenching experiments were performed in the presence or absence of 400 μM of desferoxamine. No change in the DMPO-OH signal intensity was found between these two experimental conditions. Hence, our experiments indicate that the quenching activity of α-lipoic acid is not due to its chelating activity toward metal ions (ferrous ions). It is true that the quenching ability of methyl α-lipoate is slightly lower than α-lipoic acid, and based on the ESR trapping experiment using DMPO, β-lipoic acid did not show any quenching activity toward hydroxyl radical (data not shown). All these results suggest that the disulfide bond in the thiolane ring exerts the major

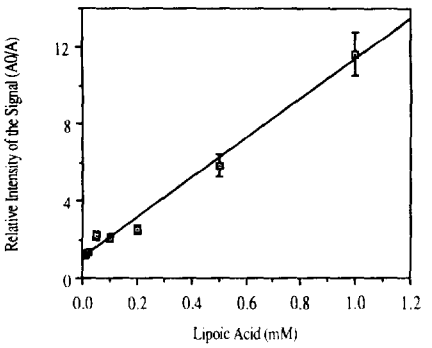


Fig. 7. Hydroxyl Radical Scavenging by α-Lipoic Acid: Determination of Rate Constant. The relative intensity of the spin adduct of DMPO and hydroxyl radical formed from the photoreaction of NP-III in the presence of various concentrations of α-lipoic acid was followed as described under Materials and Methods using final DMPO concentration of 50 mM in the reaction mixture. The Y axis shows the value of A_0 / A, where A_0 means the intensity of DMPO-OH in the absence of α-lipoic acid and A means the intensity of DMPO-OH in the presence of a variable concentration of α-lipoic acid.

quenching activity toward hydroxyl radical. The weak quenching activity of β-lipoic acid is explained by the change of the disulfide bond. We conclude from these results that α-lipoic acid is an efficient hydroxyl radical quencher owing to its unique dithiolane ring structure.

References

1. Reed, L. J. (1974) *Acc. Chem. Res.*, **7**, 40.
2. Peinado, J., Sies, H., Akerboom, T. P., (1989) *Arch. Biochem. Biophys.*, **273**, 389.
3. Kagan, V. E., Shvedova, A., Serbinova, E., Khan, S., Swanson, C., Powell, R., Packer, L. (1992) *Biochem. Pharmacol.*, **44**, 1637.
4. Kagan, V. E., Serbinova, E. A., Forte, T., Scita, G., Packer, L. (1992) *J. Lipid Res.*, **33**, 385.
5. Han, D., Handelman, G., Packer, L. (1995) *Method Enzymol.*, in press.
6. Suzuki, Y. J., Tsuchiya, M., Packer, L. (1991) *Free Radical Res. Commun.*, **15**, 255.
7. Scott, B. C., Aruoma, O. I., Evans, P. J., O'Neill, C., van der Vliet, A., Cross, C. E., Tritschler, H., Halliwell, B. (1994) *Free Rad. Res.*, **20**, 119.
8. Matsugo, S., Saito, I. (1990) *Nuc. Acid Res. Symp.*, **22**, 57.
9. Matsugo, S., Yamamoto, Y., Kawanishi, S., Sugiyama, H., Matsuura, T., Saito, I. (1991) *Angew. Chem. Int. Ed. Engl.*, **30**, 1351.
10. Matsugo, S., Kumaki, S., Mori, T., Saito, I. (1993) *Chem. Lett.*, 378.
11. Matsugo, S., Kodaira, K.-I., Saito, I. (1993) *Bioorg. Med. Chem. Lett.*, **3**, 1571.
12. Matsugo, S., Takehara, Y. Tsuruhara, T. (1992) *Photomed. Photobiol.*, **14**, 95.
13. Feix, J. B., Kalyanaraman, B. (1991) *Arch. Biochem. Biophys.*, **15**, 43.
14. Matsugo, S., Yan, L. J., Han, D., Packer L. (1994) Biochem. Biophys. Res. Commun., in press.
15. Lowry, O. H., Rosenbrough, H. J., Farr, A. L., Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
16. Yan, L. J. Traber, M. G. Packer, L. *Anal. Biochem.* submitted.
17. Finkelstein, E., Rosen, G. M., Ruckman, E. J. (1980) *J. Am. Chem. Soc.*, **102**, 4994.