a number of concerns and cautions in the interpretation of eximer images. Determination of absolute diffusion coefficients for probes that undergo intermolecular eximer formation is not possible because of uncertainties in the probe density. In addition, eximer formation requires relatively high probe densities which might perturb membrane structure. Eximer-to-monomer ratios for probes that undergo intramolecular eximer formation are independent of probe density; however, they are likely to be sensitive to the exact position of the probe in the membrane and the details of the anisotropic membrane environment (Melnick et al., 1981). Synthesis of intramolecular eximer-forming probes that can be targeted to specific membranes in intact cells would be an important advance.

In view of these limitations, the mapping of cell eximer fluorescence is a relatively simple procedure that gives information about the membrane physical state complementary to that obtained by fluorescence photobleaching recovery and anisotropy imaging techniques. Eximer mapping should have application to real-time studies of fluidity in intracellular and plasma membranes in cultured cells and intact biological tissues.

**Registry No.** PDA, 60177-21-1; DPP, 61549-24-4; pyrene, 129-00-0.

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# Ovothiols as Free-Radical Scavengers and the Mechanism of Ovothiol-Promoted NAD(P)H–$O_2$ Oxidoreductase Activity†


Tod P. Holler and Paul B. Hopkins*

*Department of Chemistry, University of Washington, Seattle, Washington 98195*

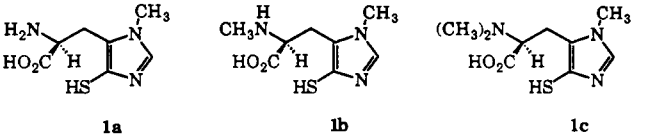


*Received June 8, 1989; Revised Manuscript Received October 9, 1989*



ABSTRACT: Racemic ovothiol A [(±)-**1a**] and the ovothiol model compound 1,5-dimethyl-4-mercaptoimidazole (DMI, **2**) were found to scavange the free radicals Fremy's salt (**4**) and Banfield's radical (**5**) much more rapidly than did the thiol antioxidant glutathione. Ovothiol A also scavenges the tyrosyl radical, with efficiency comparable to that of ascorbic acid and the tocopherol analogue trolox (**3**). The ovothiol model compound DMI was found to scavenge superoxide with a rate constant comparable to that of the reaction between superoxide and glutathione. These results suggest both a free-radical scavenging role for the ovothiols and a mechanism by which the ovothiols confer NAD(P)H–$O_2$ oxidoreductase activity upon the enzyme ovoperoxidase. Investigation of this mechanism implicates the ovothiol thiyl radical and the NAD radical as key intermediates. The ovothiyl radical appears to be unreactive toward oxygen but highly reactive toward NADH. An estimate of the one-electron oxidation potential of the ovothiol anion is presented. The physical basis for the stability of the ovothiol free radical is discussed.


The ovothiols (**1a–c**) are a family of 4-mercaptohistidine derivatives present in the eggs of a variety of marine invertebrates. Ovothiols A and C (**1a** and **1c**), and the parent compound 4-mercaptohistidine, were first isolated (Palumbo et al., 1984; Rossi et al., 1985) from the eggs of echinoderms and molluscs. Turner et al. (1987) subsequently isolated ovothiol B (**1b**) and corrected an error in the earlier structure elucidation work. Studies of the ovothiol content of various tissues (Turner et al., 1987; Rossi et al., 1985) revealed that



**1a** **1b** **1c**


† This work was supported by the Searle Scholars Program and the National Institutes of Health (GM 35466). P.B.H. is a Sloan Fellow (1988–1992) and an NIH Research Career Development Award recipient (AG 00417).



0006-2960/90/0429-1953$02.50/0
 

the presence of significant quantities of ovothiols is limited to the egg and ovary of these organisms, where its concentration may well exceed that of any other small organic molecule. The biochemical role, if any, of these remarkably abundant thiols in the life of the egg or embryo remains uncertain.

The first suggestion of a biochemical role for the ovothiols arose from investigations into the source of hydrogen peroxide required for the postfertilization hardening of sea urchin eggs' fertilization envelope (Turner et al., 1985). Hardening results from the oxidative cross-linking of tyrosine residues in envelope proteins and protects the embryo from, among other things, fatal polyspermy. The cross-linking reaction requires hydrogen peroxide and is catalyzed by a secreted peroxidase, ovoperoxidase (Deits et al., 1984). The discovery that, of all the natural thiols examined, only ovothiols could confer upon ovoperoxidase the ability to reduce $O_2$ by using NAD(P)H[1] suggested both a source for the hydrogen peroxide and a critical role for the ovothiols in the eggs' development. Subsequent observations (Turner et al., 1987), particularly the absence of ovothiols outside the egg, where the cross-linking occurs, reduced enthusiasm for the proposal but left a mechanistic puzzle: How do the ovothiols confer NAD(P)H oxidase activity on ovoperoxidase?

Cognizant of the dangers imposed upon the cell contents by the production of large quantities of hydrogen peroxide during hardening, Shapiro and co-workers suggested (Turner et al., 1987; Shapiro & Turner, 1988) that ovothiols participate in defending the contents of the egg against hydrogen peroxide. They have demonstrated (Turner et al., 1988) that the ovothiol C in sea urchin eggs is involved in the predominant mechanism for metabolism of exogenous hydrogen peroxide. The high reactivity of ovothiols toward hydrogen peroxide, 5-fold that of glutathione, is presumably important in this regard.

The high naturally occurring concentration of ovothiols in marine invertebrate eggs and the cooccurrence of significant quantities of the ubiquitous thiol glutathione prompted us to study the physical and chemical properties of ovothiols (Holler & Hopkins, 1988), comparing and contrasting them with those of glutathione. Unlike glutathione, thiol $pK_a$ 8.65, the thiol $pK_a$ of a model 4-mercaptoimidazole is 2.3. The second $pK_a$ of the 4-mercaptoimidazole, corresponding to the deprotonation of the imidazolium thiolate zwitterion, is 10.3. The mercaptoimidazole moiety of ovothiols thus exists predominantly as the imidazolium thiolate at physiological pH. The 4-mercaptoimidazole function is almost 10-fold more nucleophilic toward iodoacetamide than glutathione. Especially striking, however, is the ability of the mercaptoimidazole moiety to serve as a one-electron donor, first demonstrated by using ferricytochrome *c* as a generic one-electron acceptor. Unlike glutathione, which reacts with ferricytochrome *c* at a negligible pace in the absence of catalysis (Froede & Hunter, 1970), a model mercaptoimidazole reacted at physiological pH with a second-order rate constant of 30 $M^{-1}$ $s^{-1}$. The active reducing agent in this case is not the predominate zwitterion, but rather the anion, present at about one part in a thousand at this pH. The active reducing agent thus reacts with a second-order rate constant of roughly $10^4$ $M^{-1}$ $s^{-1}$.

The facility of one-electron donation, in conjunction with the high physiological concentration of ovothiols, raises a second issue of interest related to the postfertilization production of hydrogen peroxide. Because hydrogen peroxide and metal ions produce highly reactive oxygen-centered free radicals that can inflict insult on cellular contents, including DNA (Sies, 1983), a pressing need for scavengers of free radicals may exist in the embryo. Might ovothiols be capable of efficiently scavenging free radicals? To test this hypothesis, we have surveyed the reactivity of 4-mercaptoimidazoles toward of variety of oxygen-centered free radicals.

The facility with which the ovothiols act as one-electron donors also offers a possible explanation for the NAD(P)H–$O_2$ oxidoreductase activity of ovothiol in concert with ovoperoxidase. Like ovothiols, certain phenolic compounds confer NAD(P)H–$O_2$ oxidoreductase activity upon peroxidases, including ovoperoxidase (Turner et al., 1985). The phenol-stimulated NAD(P)H–$O_2$ oxidoreductase reaction of peroxidases is believed (Takayamo & Nakano, 1977; Michot et al., 1985) to involve heme-promoted one-electron oxidation of the phenol (eq 1), followed by reaction of the resulting

$$\text{phenol} \xrightarrow{\text{peroxidase}} \text{phenolic radical} \quad (1)$$

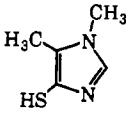
$$\text{phenolic radical} + \text{NAD(P)H} \rightarrow \text{phenol} + \text{NAD(P) radical} \quad (2)$$

$$\text{NAD(P) radical} + O_2 \rightarrow \text{NAD(P)}^+ + O_2^- \quad (3)$$

phenolic radical with NAD(P)H to yield the NAD(P) radical and return the phenol (eq 2). The transfer of one electron from the NAD(P) radical to oxygen (reaction 3) then provides $NAD(P)^+$ and superoxide, which degrades by several pathways. The phenol/phenolic radical couple thus acts as a shuttle for electrons from NAD(P)H to the heme. A reasonable mechanism by which the ovothiols might confer NAD(P)H–$O_2$ oxidoreductase activity on the heme-containing ovoperoxidase is by substituting for phenol in this process. We have therefore examined the potentially analogous reactivity of 4-mercaptoimidazole thiyl radicals with NADH and oxygen. These results also have implications in the proposed biochemical role of free-radical scavenging, since the thiyl radicals formed in the scavenging reaction might themselves prove detrimental to the cell.

## Materials and Methods

*General Procedures.* Unless otherwise specified, all reagents and enzymes were of commercial origin and were used as received. 1,5-Dimethyl-4-mercaptoimidazole (DMI, **2**) was



**2**

prepared by the method of Spaltenstein et al. (1987). Synthetic ovothiol A racemate was prepared by the published method (Holler et al., 1989). UV measurements were made on a Perkin-Elmer Model Lambda 3A UV/visible spectrophotometer (single wavelength) or a Perkin-Elmer Model 8450A diode array UV/visible spectrophotometer (dual wavelength). Unless otherwise specified, all reactions were conducted under an atmosphere of air. Thiol concentrations were determined by the method of Ellman (1958). A control experiment using a standard solution of DMI prepared by weight indicated that Ellman's assay for thiols afforded accurate and reproducible results with this compound.

*Reaction with Fremy's Salt.* HEPES-buffered solutions (ionic strength 0.05, pH 7.0, containing 0.1 mM DTPA), initially 300 $\mu$M each in antioxidant and potassium nitroso-

[1] Abbreviations: DMI, 1,5-dimethyl-4-mercaptoimidazole; DTPA, diethylenetriaminepentaacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NAD(P)H, nicotinamide adenine dinucleotide, reduced (nicotinamide adenine dinucleotide phosphate, reduced); Tris, tris(hydroxymethyl)aminomethane.

disulfonate (Fremy's salt), were monitored at 250 nm (glutathione and blank), 260 nm (trolox), 280 nm (ascorbic acid), or 320 nm (ovothiol A). When no further change in the absorbance was noted during a 5-min interval, the reaction was judged to be complete. The final change in absorbance was taken to represent 100% reaction, and the absorbances at intermediate times were scaled accordingly.

*Scavenging of Banfield's Radical.* Banfield's radical was prepared according to published procedures (Banfield & Kenyon, 1926). A stock solution (5 mM in methanol) was prepared fresh daily. Stock solutions of ascorbic acid, DMI, dithiothreitol, and glutathione were prepared in 0.1 M pH 7.0 phosphate buffer containing 1 mM DTPA; a stock solution of freshly distilled thiophenol was prepared in methanol. Kinetic runs contained an appropriate amount of antioxidant in a 1:1 mixture of absolute methanol and pH 7.0 phosphate buffer (0.1 M containing 1 mM DTPA) and were initiated by the addition of 100 $\mu$M Banfield's radical. The progress of the stirred reaction was followed at 440 nm. Pseudo-first-order rate constants were determined from plots of ln ($A_t - A_\infty$) versus time by linear regression.

*Tyrosyl Radical Scavenging.* Details of the tyrosine photooxidation assay are recorded elsewhere (Holler & Hopkins, 1989).

*Xanthine/Xanthine Oxidase Generation of Superoxide.* Xanthine oxidase (grade III, suspension in 2.3 M ammonium sulfate and 10 mM sodium phosphate buffer, pH 7.8, containing 1 mM each EDTA and sodium salicylate) and catalase (bovine liver, purified powder, 1000 units/mg) were from Sigma. Oxygen-saturated 100 mM phosphate buffer, pH 7.8, containing 250 $\mu$M xanthine, 200 $\mu$M thiol, and 20 $\mu$g/mL catalase was treated with 0.03 unit of xanthine oxidase. After 15 min at 25 °C (UV analysis of a control reaction indicated complete conversion of xanthine to urate during this time), a 20-$\mu$L aliquot of the resulting mixture was assayed for remaining thiol by using Ellman's reagent at pH 8.0 (0.1 M Tris-HCl, containing 1 mM DTPA).

*Rate Constant for the Reaction of DMI with Superoxide.* The rate constant for the reaction of 1,5-dimethyl-4-mercaptoimidazole (DMI, **2**) with superoxide was measured by the method of Asada and Kanematsu (1976), in which the thiol and epinephrine compete for superoxide generated with the xanthine/xanthine oxidase system.

*Oxidation of NADH in the Presence of Thiols.* Ferricytochrome *c* (horse heart, Sigma grade III), superoxide dismutase (bovine erythrocyte, lyophilized powder, 3000 units/mg), catalase (bovine liver, purified powder, 1000 units/mg), and NADH (preweighed vials) were from Sigma Chemical Co. Stock solutions of proteins were prepared in 10 mM phosphate buffer, pH 7.5. Thiol stock solutions were prepared in pH 8.0 HEPES-buffered water (ionic strength 0.05, containing 0.1 mM DTPA) and standardized with Ellman's reagent. Reduction of ferricytochrome *c* was monitored at 550 nm, using a $\Delta\epsilon$ of 21 000 $M^{-1}$ $cm^{-1}$ (Massey, 1959). The change in NADH concentration was monitored at 340 nm and calculated by using a $\Delta\epsilon$ of 6220 $M^{-1}$ $cm^{-1}$ for NADH, after correcting for the contribution of ferricytochrome *c* reduction at this wavelength. The $\Delta\epsilon$ (2300 $M^{-1}$ $cm^{-1}$) for ferricytochrome *c* reduction at 340 nm was determined by reducing a portion of the ferricytochrome *c* solution used in these experiments with a slight excess of ascorbic acid and dividing the change in absorbance at 340 nm by the concentration of ferricytochrome *c* reduced, determined by using $\Delta A_{550}$ and $\Delta\epsilon_{550}$ = 21 000 $M^{-1}$ $cm^{-1}$. Oxygen concentration was monitored by using a Yellow Springs Instruments Model 53 oxygen monitor equilibrated at 25 °C.

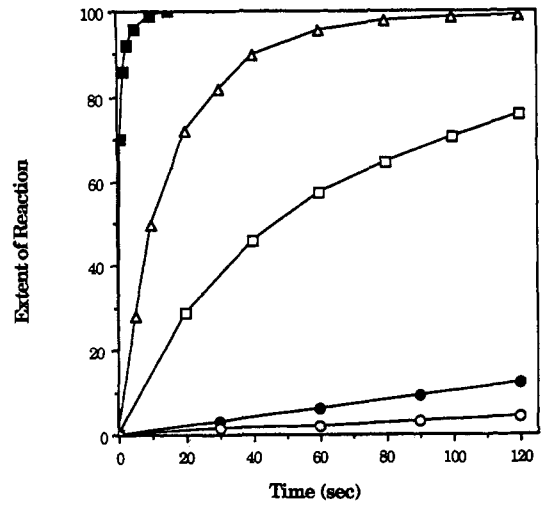
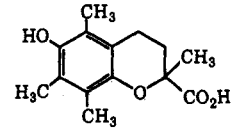



FIGURE 1: Reaction of Fremy's salt with scavengers ascorbic acid (■), trolox (**3**, △), ovothiol A (±**1a**, □), and glutathione (●); blank is denoted by (○). See Materials and Methods for experimental details.

## RESULTS

*Radical Scavenging Activity of Mercaptoimidazoles. (A) Reaction with Fremy's Salt.* To compare the ovothiols and glutathione with other, accepted free-radical scavengers, we measured the relative rates at which ovothiol A (**1a**), glutathione, ascorbic acid (vitamin C), and trolox (**3**, a water-soluble $\alpha$-tocopherol analogue) react with Fremy's salt (**4**), a stable



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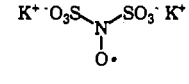

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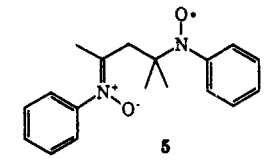

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nitroxide radical. Reactions were monitored at 250 nm for glutathione and the blank, 260 nm for trolox (**3**), 280 nm for ascorbic acid, and 320 nm for ovothiol A (**1a**). The results are presented in Figure 1. The extent of reaction represents a scaling to 100 of the absorbance change as a function of time, relative to the maximum absorbance change upon completion of the reaction. Although not as effective as trolox (**3**) or ascorbic acid, ovothiol A (**1a**) reacted with Fremy's salt considerably more rapidly than did glutathione. The identities of the products of these reactions, the stoichiometries, and the kinetics of the reactions were not rigorously evaluated.

*(B) Reaction with Banfield's Radical.* Reactions of ascorbic acid, DMI (**2**), thiophenol, dithiothreitol, and glutathione with a more stable nitroxide free radical, Banfield's radical (**5**), at pH 7.0 (phosphate) in 1:1 methanol/water were monitored at 440 nm. For all these reactions, except with ascorbic acid, which was too rapid to quantify, and thiophenol, which dislayed some curvature in the plot of observed rate constant versus thiophenol concentration, reaction with Banfield's radical was first order in both radical and reducing agent, and rate constants (Table I) could be measured by using pseudo-first-order techniques. In qualitative agreement with the Fremy's salt assay, it was found that ascorbic acid was considerably more reactive than the mercaptoimidazole, while

Table I: Rate Constants for the Reaction of Radical Scavengers with Banfield's Radical

| scavenger | rate constant ($M^{-1}$ $s^{-1}$)[a] |
|---|---|
| glutathione | 0.012 ± 0.001 |
| dithiothreitol | 0.049 ± 0.005 |
| thiophenol | 0.27 ± 0.09[b] |
| DMI | 1.55 ± 0.03 |
| ascorbic acid | too fast to measure |

[a] Rate constants are the average (±standard deviation) of pseudo-first-order experiments at three different concentrations of radical scavenger (glutathione at 20, 40, and 80 mM; dithiothreitol at 9.8, 40, and 80 mM; thiophenol at 6.5, 13, and 26 mM; and DMI at 2, 4, and 8 mM) as described under Materials and Methods. [b] This value should be taken as qualitative: the reaction is somewhat greater than first order in thiophenol, leading to the indicated high error when fitted to overall second-order kinetics.

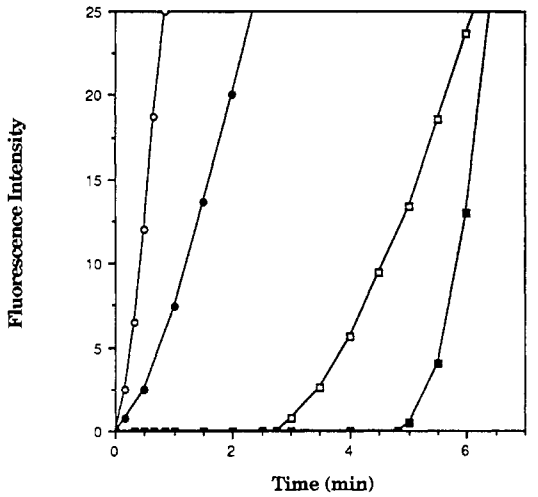



FIGURE 2: Effect of 2 μM concentrations of glutathione (●), ovothiol A (±**1a**, □), and ascorbic acid (■) upon the photochemical coupling of L-tyrosine (blank, ○). Reaction mixtures contained the indicated scavenger, 400 μM L-tyrosine, and 20 μg/mL each of superoxide dismutase and catalase in pH 7.0, 0.1 M phosphate-buffered water containing 0.1 mM DTPA. For further details see Holler and Hopkins (1989).

glutathione was much slower. Thiophenol and dithiothreitol were found to be intermediate in radical scavenging activity between the mercaptoimidazole and glutathione.

(*C*) *Suppression of Tyrosine Photooxidation.* The relative activity of several reducing agents as scavengers of photogenerated tyrosyl radicals was evaluated by monitoring their ability to suppress formation of the fluorescent 3,3′-tyrosine dimers (Holler & Hopkins, 1989). To minimize the influence of superoxide and hydrogen peroxide (formed by scavenging of the photoejected electron by oxygen, followed by dismutation of the resulting superoxide), 20 μg/mL each of catalase and superoxide dismutase were included in the reactions. Once again, the mercaptoimidazole-bearing ovothiol A (**1a**) was significantly superior to glutathione, which was only weakly inhibitory of fluorescence buildup (Figure 2). Ovothiol A (**1a**) completely inhibited the appearance of fluorescence for a period of time proportional to its initial concentration. At an equivalent concentration, ascorbic acid delayed the appearance of appreciable fluorescence for twice as long as ovothiol A.

(*D*) *Superoxide Scavenging.* As a preliminary assay for the reactivity of mercaptoimidazoles toward superoxide, ovothiol A was exposed to 2 molar equiv of superoxide, generated by use of the xanthine/xanthine oxidase system, at pH 7.8, in the presence of 20 μg/mL catalase. The reaction mixture was then assayed for unreacted thiol by using Ellman's reagent. For comparison, an identical experiment was conducted with

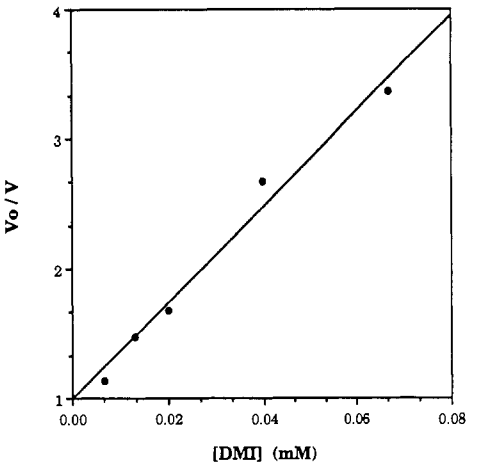



FIGURE 3: Competition between 500 μM epinephrine and indicated concentrations of DMI (**2**) for enzymatically generated superoxide radical. Reactions were conducted in 0.1 M phosphate buffer (pH 7.8, containing 1 mM DTPA) saturated with oxygen, and containing 250 μM xanthine and 25 μg/mL catalase. Reactions were initiated by addition of xanthine oxidase (0.03 unit, Sigma). Rates were determined by measuring the change in absorbance at 440 nm between 1 and 3 min after initiation.

glutathione in place of ovothiol A. It was found that 17% of the ovothiol A and 5% of the glutathione were consumed by the superoxide, values insignficantly different given the crude nature of the assay. A more quantitative measure of the reactivity was achieved by the method of Asada and Kanematsu (1976), in which the ability of a substance to suppress the reaction of epinephrine with superoxide (again generated by use of the xanthine/xanthine oxidase system at pH 7.8) is used to calculate the rate constant for the reaction of the substance with superoxide. The relative velocity of epinephrine oxidation was determined by measuring the change in absorbance between 1 and 3 min after the initiation of the reaction. A plot of the relative rates of epinephrine oxidation versus the concentration of DMI present is shown in Figure 3. The slope of the line in Figure 3 (0.037 $\mu M^{-1}$), the concentration of epinephrine used (500 μM), the rate constant for the reaction of superoxide with epinephrine ($4 \times 10^4$ $M^{-1}$ $s^{-1}$), and eq 4 yielded a second-order rate constant of $7.4 \times 10^5$ $M^{-1}$ $s^{-1}$ for the reaction of DMI with superoxide at pH 7.8. Under these same conditions, glutathione and superoxide react with a rate constant of $6.7 \times 10^5$ $M^{-1}$ $s^{-1}$ (Asada & Kanematsu, 1976).

$$V_0/V_{DMI} = 1 + k_{DMI}[DMI]/k_{epinephrine}[epinephrine] \quad (4)$$

(*E*) *Mercaptoimidazole-Catalyzed Oxidation of NADH by Ferricytochrome c.* The direct reduction of ferricytochrome *c* by NADH, at pH 8.0, under air, at 25 °C, is sluggish (Figure 4). In contrast, addition of 13 mol % DMI (Figure 4A) or ovothiol A (data not shown) resulted in a rapid redox reaction, yielding $NAD^+$ and ferrocytochrome *c*, as indicated by the UV spectrum of the product mixture. The stoichiometry of the reaction under these conditions, determined by quantitative evaluation at 550 nm (ferricytochrome *c*) and 340 nm (NADH), was found to be approximately 2 mol of ferricytochrome *c* reduced per mole of NADH oxidized (Figure 5). In agreement with this was the absence of any consumption of oxygen during this reaction, as monitored with an oxygen electrode (Figure 6). The increase in absorbance at 550 nm (ferricytochrome *c* reduction) was subjected to first-order analysis. A plot of ln $(A_\infty - A_t)$ versus time was linear for the first half-life. Assuming that the thiol concentration is unchanged during the course of the reaction, the

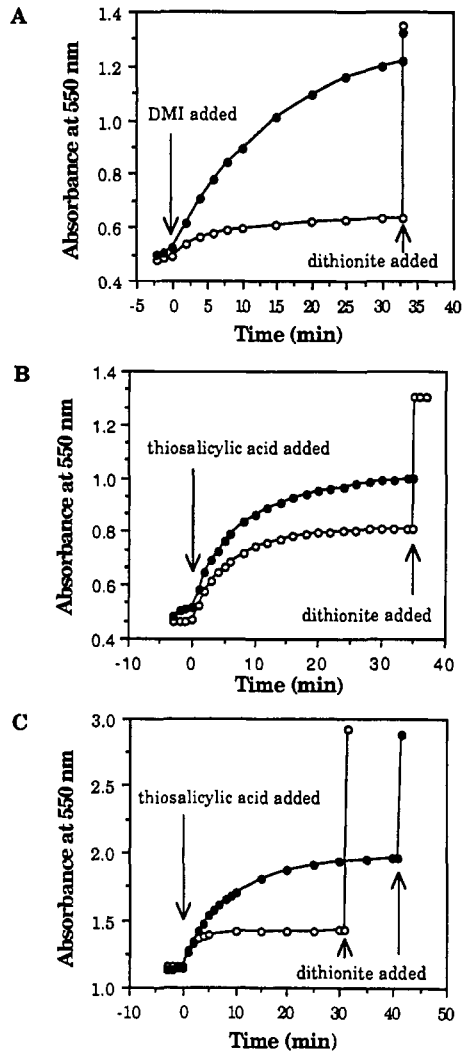



FIGURE 4: Reduction of horse heart ferricytochrome *c* by NADH. (A) Mediated by DMI (**2**) under atmosphere of air; (○) mixture contains only ferricytochrome *c* (40 μM) and DMI (5 μM); (●) mixture contains ferricytochrome *c* (40 μM), DMI (5 μM), and NADH (360 μM). (B) Mediated by thiosalicylic acid (**6**) under atmosphere of air; (○) mixture contains ferricytochrome *c* (40 μM) and thiosalicylic acid (13 μM); (●) mixture contains ferricytochrome *c* (40 μM), thiosalicylic acid (13 μM), and NADH (360 μM). (C) Mediated by thiosalicylic acid (**6**) under a predominately argon atmosphere; (○) mixture contains ferricytochrome *c* (84 μM) and thiosalicylic acid (15 μM); (●) mixture contains ferricytochrome *c* (84 μM), thiosalicylic acid (15 μM), and NADH (425 μM).

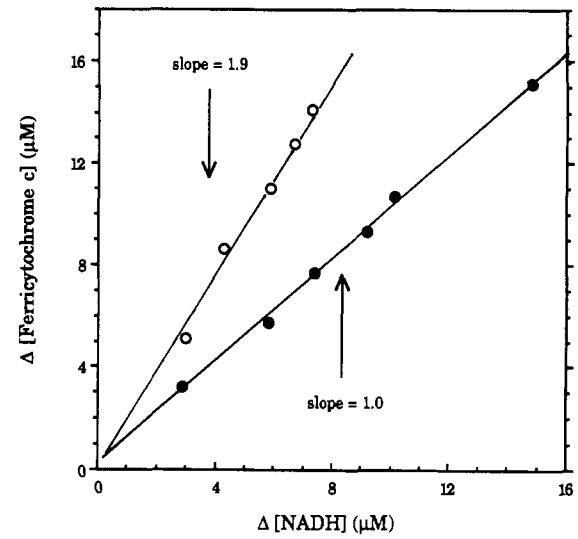



FIGURE 5: Stoichiometry of the DMI-catalyzed reduction of horse heart ferricytochrome *c*. (○) Reaction mixture contains 20 μM ferricytochrome *c*, 5 μM DMI (**2**), and 400 μM NADH. A line of slope 1.9 is obtained by linear regression analysis. (●) Reaction mixture contains 20 μM ferricytochrome *c*, 5 μM DMI, 400 μM NADH, and 20 μg/mL each of superoxide dismutase and catalase. A line of slope 1.0 is obtained by linear regression analysis.

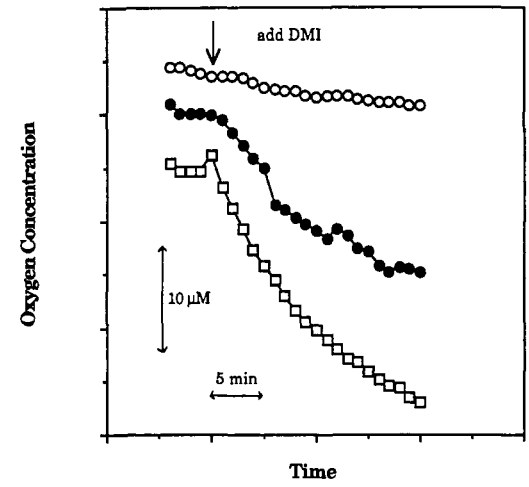


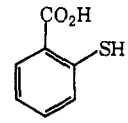

FIGURE 6: Consumption of oxygen during the DMI-catalyzed reduction of horse heart ferricytochrome *c* by NADH. Mixture contains 100 μM ferricytochrome *c*, 10 μM DMI (**2**), 400 μM NADH, and either (○) no additives, (●) 20 μg/mL each superoxide dismutase and catalase, or (□) 20 μg/mL superoxide dismutase.

depletion of ferricytochrome *c* proceeds with a rate constant of 480 $M^{-1}$ $s^{-1}$, approximately twice that previously observed for the direct reaction of DMI with ferricytochrome *c* at pH 8.0 (Holler & Hopkins, 1988).

The role of oxygen in the mercaptoimidazole-catalyzed reduction of ferricytochrome *c* by NADH was probed by conducting the reaction in the presence of superoxide dismutase and catalase. Addition of 20 μg/mL each of superoxide dismutase and catalase effected both the rate and the stoichiometry of the process. The ratio of ferricytochrome *c* reduced to NADH oxidized became 1:1 (Figure 5). The rate of ferricytochrome *c* consumption was still first order in cytochrome *c*. Changing the concentration of DMI (Figure 7) demonstrated a first-order dependence of this rate on DMI. Assuming no change in the thiol concentration during the reaction, the second-order rate constant was 245 $M^{-1}$ $s^{-1}$, matching that previously observed for the direct reaction of ferricytochrome *c* and DMI (Holler & Hopkins, 1988). At concentrations in excess of ca. 100 μM NADH, the reaction was zero order in this component; below this level, the change in absorbance at 550 nm deviated from first-order behavior (Figure 7). Under these conditions, oxygen was consumed at an appreciable rate; omitting catalase approximately doubled the rate of oxygen consumption (Figure 6).

The ability of other thiols to catalyze the reaction of ferricytochrome *c* by NADH was examined. Glutathione, which reacts very slowly with ferricytochrome *c*, did not catalyze the reaction (data not shown). Addition of 33 mol % thiosalicylic acid (**6**) did result in the reduction of ferricytochrome *c* (Figure 4B), but the extent of reaction could be accounted for almost completely by the direct reactions of the thiol, and of NADH, with ferricytochrome *c*. Partial degassing, by bubbling argon through the reaction buffer prior to addition of cytochrome *c* and thiol, resulted in a modest catalysis of the redox process by thiosalicylic acid (Figure 4C).

The oxidations of DMI (**2**) and thiosalicylic acid (**6**) with potassium ferricyanide under air at pH 8.0, both of which are complete within a few minutes at 25 °C as indicated by UV



6

analysis, were monitored in the oxygen electrode. It was found that ferricyanide oxidation of thiosalicylic acid (**6**) results in appreciable uptake of oxygen (Figure 8), while the corresponding oxidation of DMI did not. The stoichiometry of these reactions was determined by oxidizing each thiol with a deficiency of ferricyanide and determining the remaining thiol with Ellman's reagent. Consistent with the results of the oxygen electrode, oxidation of DMI had a stoichiometry of 1 mercaptoimidazole oxidized per ferricyanide reduced, while 2 thiosalicylic acids were oxidized by a single ferricyanide (Figure 9). From this we infer that the remaining oxidizing equivalents consumed by thiosalicylic acid must be supplied by oxygen.

## Discussion

*Ovothiols as Free-Radical Scavengers.* Repair of free radicals is believed to be the mechanism by which thiols serve as radioprotective agents (Ormerod & Alexander, 1963; Baker et al., 1982). Ross et al. (1985) have found that glutathione scavenges free radicals formed by the peroxidase-catalyzed oxidation of xenobiotics. Stock et al. (1986) have proposed a mechanism of glutathione conjugate formation that is dependent upon one-electron oxidation of glutathione. Although these processes can be seen as beneficial to the cell, glutathione thiyl radicals react rapidly with oxygen (Quintiliani et al., 1977), and this oxygen activation may lead to the oxidative damage of other physiological substrates (Ortiz de Montellano & Grab, 1986).

Because oxygen-centered radicals react faster with thiolates than with thiols (Simic & Hunter, 1986), ovothiols, which exist primarily as the thiolate at physiological pH, might exceed glutathione as scavengers of oxygen-centered free radicals. Glutathione and 4-mercaptoimidazoles were distinct in their reactivity toward model oxygen-centered free radicals. Using the reaction of Fremy's salt (Zimmer et al., 1971) with a variety of reductants, we have evaluated radical scavenging activity. On the basis of spectroscopic changes that occurred upon admixture of potential scavengers with Fremy's salt (**4**), a wide range of reaction rates was observed (Figure 1). The radical scavenging activity order ascorbic acid > trolox (**3**) > ovothiol A [(±)-**1a**] > glutathione was thus established.

Banfield's radical (**5**; Banfield & Kenyon, 1926) is, like Fremy's salt, a nitroxide free radical. The reactions of potential scavengers with Banfield's radical were investigated. The results (Table I) reflect an ordering of reactivity: ascorbic acid ≫ DMI (**2**) > thiophenol > dithiothreitol > glutathione. Notably, a 2 order of magnitude difference separates glutathione and the ovothiol model.

The tyrosyl radical is a likely ultimate product in the cascade of reactions initiated by such highly reactive particles as the hydroxyl radical (Davies, 1987). Because tyrosyl radicals, generated by using the incident radiation of a standard fluorometer, undergo dimerization to fluorescent 3,3′-dityrosine dimers, the scavenging of the radicals is readily evaluated by suppression of the appearance of fluorescence (Holler & Hopkins, 1989). In qualitative agreement with the other assays, the ovothiol analogue DMI was also superior to glutathione as a scavenger of tyrosyl radicals (Figure 2).

Superoxide is generated by several cellular processes and, in conjunction with hydrogen peroxide and metal ions, poses

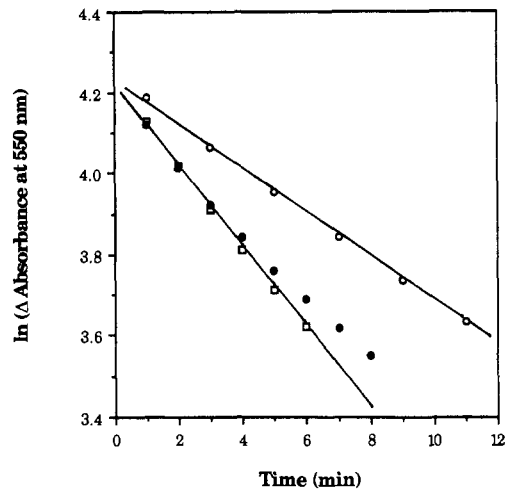



FIGURE 7: First-order analysis of the reduction of ferricytochrome *c*, monitored at 550 nm. (○) Reaction mixture contains 40 μM ferricytochrome *c*, 4.9 μM DMI, and 400 μM NADH. (●) Reaction mixture contains 40 μM ferricytochrome *c*, 9.8 μM DMI, and 200 μM NADH. (□) Reaction mixture contains 40 μM ferricytochrome *c*, 9.8 μM DMI, and 50 μM NADH. All reactions contained 10 μg/mL superoxide dismutase and catalase. Lines have been shifted vertically for better presentation.

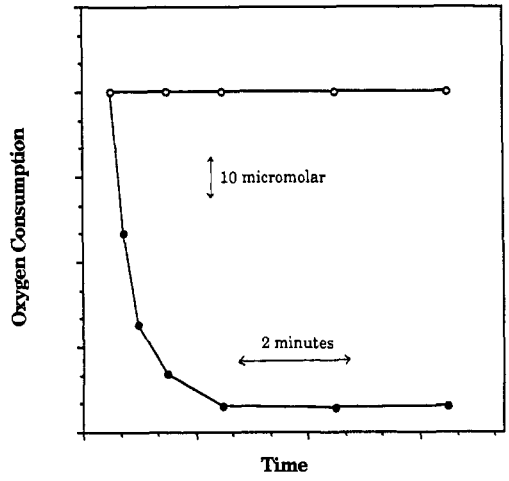



FIGURE 8: Consumption of oxygen during ferricyanide oxidation of thiols at pH 8.0. Thiyl radicals were generated by adding potassium ferricyanide solution (300 μM final concentration) to a solution of the thiol (500 μM final concentration) in pH 8.0 buffer ($I$ = 0.05 HEPES, 0.1 mM DTPA). (○) DMI (**2**), ovothiol A [(±)-**1a**], or blank; (□) thiosalicylic acid (**6**).

a threat to cellular structures (Fridovich, 1978). Despite the relatively modest rate constant for the reaction of glutathione with superoxide, the overwhelming intracellular concentration of glutathione may allow it to compete with superoxide dismutase as a superoxide scavenger (Wefers & Sies, 1983). Direct comparison of the reactivity of ovothiol A and glutathione toward an identical quantity of superoxide revealed only a slight difference in reactivity. Second-order rate constants were found to be nearly identical. The latter suggests that the rates of reaction of superoxide with thiols depend upon factors other than one-electron donating ability, such as metal ion mediated processes (Fee, 1980; Thomas et al., 1988). It appears unlikely that the ovothiols play any in vivo role in superoxide scavenging beyond that normally associated with cysteine-derived thiols.

*On the Mechanism of the NADH–Oxidoreductase Reaction.* In the presence of certain catalysts, including phenols, heme-containing peroxidases may act as NAD(P)H–$O_2$ oxidoreductases (Akazawa & Conn, 1958; Takanaka & O'Brien,

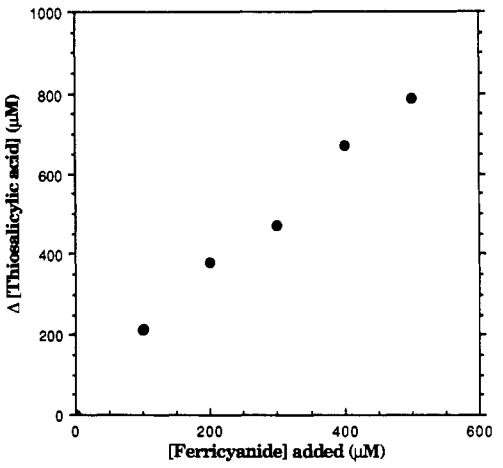



FIGURE 9: Aerobic oxidation of thiosalicylic acid by ferricyanide. Aliquots of potassium ferricyanide solution (10 mM) were added to a solution of thiosalicylic acid in pH 8.0 (0.1 M Tris-HCl, 1 mM DTPA) buffered water. The initial concentration of thiosalicylic acid was 900 μM in all cases. Ten minutes after addition of ferricyanide, the concentration of thiol remaining was determined by using Ellman's reagent. The change in thiol concentration is calculated by subtracting the thiol remaining from the value obtained with no ferricyanide added.

1975; Klebanoff, 1962). These reactions appear to involve free radicals (Takayamo & Nakano, 1977; Michot et al., 1985). Phenol apparently acts as a one-electron donor (eq 1), generating a phenolic radical that in turn participates in the one-electron oxidation of NADH (eq 2). A similar mechanism might explain the action of ovothiols in conjunction with ovoperoxidase as a catalyst for the air oxidation of NADH. This was tested by evaluating the ability of 4-mercaptoimidazole radicals, generated by the one-electron oxidation of DMI (**2**) with ferricytochrome *c*, to oxidize NADH.

In the absence of a suitable catalyst, NADH does not reduce ferricytochrome *c* at an appreciable rate. Addition of 13 mol % DMI initiated a reaction with the stoichiometry 2 mol of ferricytochrome *c* reduced by 1 mol of NADH to afford ferrocytochrome *c* and $NAD^+$ (Figure 4). The depletion of ferricytochrome *c* in the DMI-catalyzed reaction is first order in both cytochrome and thiol and, assuming that the thiol concentration remains unchanged during the reaction, has a rate constant of 480 $M^{-1}$ $s^{-1}$, twice that observed for the direct reaction of DMI with ferricytochrome *c*. These observations are consistent with the reaction proceeding via the elementary steps shown in eqs 5–8. We propose that rate-determining

$$\text{cyt } c \text{ (oxidized)} + \text{DMI} \rightarrow \text{cyt } c \text{ (reduced)} + \text{DMI thiyl radical} \quad (5)$$

$$\text{DMI thiyl radical} + \text{NADH} \rightarrow \text{DMI} + \text{NAD radical} \quad (6)$$

$$\text{NAD radical} + O_2 \rightarrow NAD^+ + O_2^{\bullet -} \quad (7)$$

$$O_2^{\bullet -} + \text{cyt } c \text{ (oxidized)} \rightarrow O_2 + \text{cyt } c \text{ (reduced)} \quad (8)$$

reaction of ferricytochrome *c* and thiol affords the thiyl radical (eq 5), which in turn oxidizes NADH to the NAD radical (eq 6). In the case of the glutathione thiyl radical, the latter reaction occurs via an electron transfer rather than hydrogen atom transfer and has a rate constant of $2.3 \times 10^8$ $M^{-1}$ $s^{-1}$. The failure of the DMI-catalyzed reduction of ferricytochrome *c* by NADH to follow pseudo-first-order kinetics when the NADH concentration is lower than ca. 200 μM suggests that the DMI thiyl radical can react by pathways other than eq 6, for example, dimerization to the disulfide. The NAD radical (Willson, 1970) reduces molecular oxygen with a rate constant of $1.9 \times 10^9$ $M^{-1}$ $s^{-1}$, producing $NAD^+$ and superoxide anion (eq 7). The superoxide anion reduces ferricytochrome *c* with a rate constant of $2.6 \times 10^5$ $M^{-1}$ $s^{-1}$ (Butler et al., 1982) (eq 8). The latter reaction accounts for the reduction of 2 equiv of ferricytochrome *c* (eqs 5 and 8) per rate-limiting reaction of thiol with ferricytochrome *c* and the observed rate which is twice that of the direct reaction of the ferricytochrome and thiol. This scheme accounts for the catalytic action of the mercaptoimidazole and, in the absence of superoxide dismutase, for the observed stoichiometry of 2 ferricytochromes *c* per NADH and the lack of overall oxygen consumption.

Inclusion of superoxide dismutase and catalase alters the rate and the stoichiometry of the mercaptoimidazole-catalyzed reaction of ferricytochrome *c*, with eq 9 replacing eq 8 as the

$$4O_2^{\bullet -} + 4H^+ \xrightarrow{\text{superoxide dismutase, catalase}} 3O_2 + 2H_2O \quad (9)$$

ultimate step, diverting one electron from NADH to oxygen rather than ferricytochrome *c* (a change in stoichiometry to 1 ferricytochrome *c* per NADH), and the net uptake of oxygen.

The failure of glutathione to catalyze the reaction of ferricytochrome *c* with NADH affords further support for this mechanistic picture. Glutathione reacts at an insignificant rate with ferricytochrome *c* in the absence of catalysts (Massey et al. 1971) and thus fails to replace ovothiol in eq 5. Whether the ovoperoxidase is capable of oxidizing glutathione is unknown. This may not be relevant, however, because ability to generate a thiyl radical is insufficient to guarantee the operation of mechanistic steps 6 and 7. For example, thiosalicylic acid, which does react with ferricytochrome *c* (Figure 4), failed to catalyze the reaction with NADH. It seems likely that the reaction of ferricytochrome *c* with thiosalicylic acid produces a thiyl radical. This radical, for reasons we have not clarified, fails to support the catalytic cycle. Instead, the radical, or some other intermediate, reacts with oxygen in a manner that precludes net reaction with NADH. Several observations support this hypothesis. Oxidation of DMI and that of thiosalicylic acid with ferricyanide under air at 25 °C have different stoichiometries. In the former case, negligible $O_2$ was consumed (Figure 8) and a 1:1 ratio of ferricyanide and thiol are consumed. In the latter case, oxygen was rapidly consumed (Figure 8), and the ratio becomes 1:2. Furthermore, when oxygen was partially excluded from a mixture of NADH, ferricytochrome *c*, and thiosalicylic acid, a degree of catalysis was observed (Figure 4C). It thus appears likely that it is the lower reactivity of the mercaptoimidazole radical which is responsible for its survival to react according to eq 6.

The pathway described by eqs 5–8 need not constitute the sole mechanism for NADH oxidation in more complex systems. Yakota and Yamazaki (1977) have provided evidence that a radical chain process involving NADH and superoxide anion can result in the air oxidation of NADH.

Turner et al. (1985) observed significant catalysis of the NAD(P)H oxidase activity by manganese ions ($Mn^{2+}$). This catalysis may be caused by a combination of reactions 10 and 11 (Thomas et al., 1988), in combination with reactions 6 and

$$Mn^{2+} + O_2^{\bullet -} \rightarrow Mn^{3+} + H_2O_2 \quad (10)$$

$$Mn^{3+} + RS^- \rightarrow Mn^{2+} + RS^{\bullet} \quad (11)$$

7. These reactions constitute a free-radical chain process, catalytic in both manganese and thiol, in which oxygen is reduced to hydrogen peroxide while NADH is oxidized to $NAD^+$.

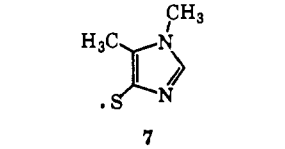
## Conclusion

The ovothiols bear a sulfur substituent which is more redox reactive than that in the ubiquitous thiol glutathione. Turner

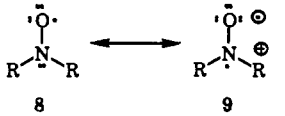
et al. (1985, 1986) have reported that ovothiols uniquely confer NAD(P)H–$O_2$ oxidoreductase activity upon the heme-containing ovoperoxidase and (Turner et al., 1988) that the ovothiols can detoxify exogenous hydrogen peroxide in the sea urchin egg. We demonstrate here that there are significant differences in the ability of ovothiols and glutathione to serve, formally, as one-electron donors and thus form thiyl radicals. This reactivity accounts for the role of ovothiols as oxidoreductase activity initiators and suggests a role for the ovothiols as biological free-radical scavengers. We have not directly observed the thiyl radical of ovothiol or DMI, nor have we conclusively proven that the reactions of ovothiols proceed through electron transfer. We infer the intermediacy of radicals and the transfer of electrons as the mechanism of their formation on the basis of the data presented here.

Why are the ovothiols more reactive than glutathione as formal one-electron donors? This kinetic difference might reflect a thermodynamic advantage. The oxidation potential of the glutathione anion ($RS^- \rightarrow RS^\bullet + e^-$) is estimated to be −0.80 to −1.1 V (Surdhar & Armstrong, 1986). The corresponding value for 1,5-dimethyl-4-mercaptoimidazole, a model for ovothiols, can be estimated, on the basis of the reduction potential of ferricytochrome *c* (−0.26 V), the forward rate constant for the reaction of ferricytochrome *c* and the 1,5-dimethyl-4-mercaptoimidazolate anion ($10^4$ $M^{-1}$ $s^{-1}$), which presumably reflects rate-controlling electron transfer, and the conservative assumption that the reverse reaction is slower than diffusion control ($10^{10}$ $M^{-1}$ $s^{-1}$). As such, the equilibrium constant for eq 5 must be greater than ($10^4$ $M^{-1}$ $s^{-1}$)/($10^{10}$ $M^{-1}$ $s^{-1}$), or $10^{-6}$, corresponding to an $E^\circ = -0.35$ V. The half reaction $DMI^- \rightarrow DMI^\bullet + e^-$ is thus estimated to have a potential greater than −0.35 + (−0.26) = −0.61 V. The less conservative assumption that the known rate at which glutathione thiyl radical oxidizes ferrocytochrome *c*, $10^8$ $M^{-1}$ $s^{-1}$ (Forni & Willson, 1986), is a more likely value for the corresponding reaction of ovothiol thiyl radical leads to an even more favorable estimate of −0.49 V. Ovothiol anion is thus estimated to possess 0.19–0.51-V thermodynamic advantage over glutathione thiolate as a one-electron donor. Adjustment of these values to reflect ionization states at physiological pH diminishes by 0.1 V the advantage of a 4-mercaptoimidazole ($pK_a$ 10.3) relative to glutathione ($pK_a$ 8.6). We speculate that the latter is reflected in the relatively rapid kinetics of ovothiol oxidation reactions.

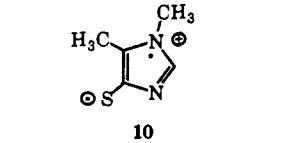
We speculate that unusual stability of the radical **7** is responsible for the superiority of the mercaptoimidazole nucleus as a one-electron donor. Delocalization of the electron deficiency over the adjoining aromatic ring as in phenolic radicals is no doubt involved. Additional stabilization may result from the special electronic relationship of the thiyl and imidazole ring, best illustrated by analogy to the highly stable nitroxide radical, with resonance forms **8** and **9**. Although diminished by the lower electronegativity of sulfur, a similar charge-separated form **10** may contribute to the stability of imidazole thiyl radicals. Danen and Newkirk (1976) have similarly rationalized the remarkable stability of thionitroxide radicals (**11** ↔ **12**).



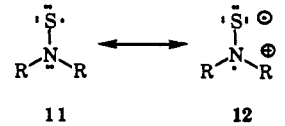
**7**



**8** **9**



**10**



**11** **12**

## Acknowledgments

We thank B. M. Shapiro, E. Turner, and J. Heinicke for numerous discussions and disclosure of results prior to publication.

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# The LexA Repressor and Its Isolated Amino-Terminal Domain Interact Cooperatively with Poly[d(A-T)], a Contiguous Pseudo-Operator, but Not with Random DNA: A Circular Dichroism Study†

Serge Hurstel, Michèle Granger-Schnarr, and Manfred Schnarr*

*Institut de Biologie Moléculaire et Cellulaire, CNRS LP 6201 Affiliated to INSERM and Université Louis Pasteur, 15 rue René Descartes, 67084 Strasbourg, France*

*Received July 10, 1989; Revised Manuscript Received October 3, 1989*

ABSTRACT: The interaction of the entire LexA repressor and its amino-terminal DNA binding domain with poly[d(A-T)] and random DNA has been studied by circular dichroism. Binding of both protein species induces an about 2-fold increase of the positive circular dichroism band at about 270 nm of both polynucleotides, allowing a precise determination of the principal parameters as a function of mono- and divalent salt concentration and pH. Both proteins interact much more strongly (about 2000-fold) with poly[d(A-T)] than with random DNA as expected from the homology with the specific consensus binding site of LexA (CTGTATATATATACAG). For both LexA and its DNA binding domain we find that the interaction with poly[d(A-T)] is cooperative with a cooperativity factor $\omega$ of about 50–70 for both proteins over a wide range of solvent conditions, suggesting that the carboxy-terminal domain of LexA is not involved in this type of cooperativity. On the contrary, no cooperativity could be detected for the interaction of the LexA DNA binding domain with a random DNA fragment. The overall binding constant $K\omega$ (or simply $K$ in the case of random DNA) depends strongly on the salt concentration as observed for most protein–DNA interactions, but the behavior of LexA is unusual in that the steepness of this salt dependence ($\delta \log K\omega/\delta \log [NaCl]$) is much more pronounced at slightly acidic pH values as compared to that at neutral or slightly alkaline pH. The behavior is not easily understood in terms of the current theories on the electrostatic contribution to protein–DNA interactions on the basis of polyelectrolyte theory. A comparison of the overall binding constant $K\omega$ of the entire LexA repressor and its DNA binding domain reveals that LexA binds only 20–50-fold stronger under a wide variety of salt and pH conditions. This result tends to demonstrate further that the additional energy due to the dimerization of LexA via the carboxy-terminal domain should be rather weak as expected from the small dimerization constant of LexA ($2 \times 10^{-4}$ $M^{-1}$).

The SOS network of *Escherichia coli* consists of a family of about 20 unlinked genes involved mainly in DNA repair of damages caused to DNA by chemical and radiative carcinogens. These SOS genes include those responsible for excision repair of DNA adducts (uvrA,uvrB, uvrD), repair by recombination (recA, recN, and probably ruv), and SOS mutagenesis (umuDC, mucAB, recA) as well as inhibition of cell division (sulA) [for reviews see Little and Mount (1982) and Walker (1984)]. In the absence of DNA damage the expression of these genes is inhibited by the LexA repressor, a protein of 202 amino acids, which consists of two structural domains linked by a flexible "hinge" region (Little & Hill, 1985).

† This work was supported by research grants from the European Community (ST2J-0291), from INSERM (871 007), from the Ligue Nationale de la Lutte contre le Cancer and the Comité Départmental du Haut-Rhin, and from the Association pour la Recherche sur le Cancer.
* To whom correspondence should be addressed.

0006-2960/90/0429-1961$02.50/0 