Mechanism of Thyroxine-Mediated Oxidation of Reduced Nicotinamide Adenine Dinucleotide in Peroxidase–H_2O_2 System†

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ABSTRACT: The oxidation of reduced nicotinamide adenine dinucleotide (NADH) by the horseradish peroxidase (HRP)-H_2O_2 system is greatly increased by the addition of thyroxine or related compounds. On the basis of a study of the rate of NADH oxidation in the presence of various concentrations of thyroxine, it is clear that thyroxine acts as a catalyst for NADH oxidation. Spectral changes of a HRP-H_2O_2 complex (compound I) indicate that thyroxine acts as an electron donor to both compounds I and II. The rate of electron donation from thyroxine is much faster than that from NADH. The HRP-H_2O_2 system requires 0.83 mol of O_2 for the oxidation of 1 mol of NADH. Ferricytochrome *c* is reduced to ferrocytochrome *c* by the system, and causes an inhibition of O_2 consumption which can be abolished by superoxide dismutase. Judging from the inhibition of O_2 uptake by ferricytochrome *c*, about 54% of the total flux of electrons from NADH to oxygen appears to proceed by way of $O_2^{-}\cdot$. These results suggest that the initial step of thyroxine-mediated NADH oxidation by HRP and H_2O_2 is the formation of oxidized thyroxine, a phenoxy radical, which attacks NADH to produce NAD·.

HRP[1] (EC 1.11.1.7 donor:H_2O_2 oxidoreductase) catalyzes the oxidation of compounds by hydrogen peroxide (Chance, 1952; George, 1952; Cotton et al., 1973). Various peroxidase preparations iodinate tyrosine with the formation of iodinated analogues (in the presence of H_2O_2), which are precursors of thyroid hormone synthesis (Kondo, 1961; Coval and Taurog, 1967; Jirousek and Pritchard, 1970; Björksten, 1970). However, the HRP-H_2O_2 system can also oxidize free thyroxine to form inorganic iodide and diiodotyrosine (Björksten, 1966).

On the other hand, Klebanoff (1959a,b; 1960; 1961; 1962a,b) has shown that thyroxine and structurally related compounds catalyze the oxidation of NADH by peroxidase (HRP and myelo- and lactoperoxidases) in the presence of H_2O_2. Jolin and Morreale de Escobar (1971) have demonstrated that removal of the β-phenolic ring iodine atoms from thyroxine in a HRP-H_2O_2 system is delayed by NADH until most of NADH has been oxidized. Little is known, however, of the mechanism of thyroxine-mediated NADH oxidation by a peroxidase-H_2O_2 system.

The present work was undertaken to identify the mechanism of thyroxine-mediated NADH oxidation by HRP-H_2O_2.

Materials and Methods

Chemicals. L-[3′,5′-^{131}I]Thyroxine and L-thyroxine were obtained from Tokyo Daiichi Seiyaku and Sigma Chemical Co., respectively. 2,4,6-Triiodophenol was purchased from Wako Chemical Co. 3,5-Diiodo-L-thyronine and DL-thyronine were obtained from Mann Research Laboratories and Aldrich Chemical Co., respectively. 3,5,3′-Triiodo-L-thyronine, 3,5-diiodo-3′-isopropyl-DL-thyronine, 3,5,3′,5′-tetraiodothyropropionic acid, and 3,5,3′-triiodothyroacetic acid were kindly supplied by Smith Kline and French Lab. 2,6-Diiodohydroquinone was a gift from Dr. G. Cilento. 3′,5′-Diiodo-DL-thyronine was prepared by the iodination of DL-thyronine and purified by paper chromatography in *tert*-amyl alcohol saturated with 6 N NH_4OH (Roche et al., 1959). The methyl ester of L-thyroxine was prepared by the treatment of L-thyroxine with dry HCl in absolute methanol. 2,6-Diiodo-4-nitrophenol (Burger and Wilson, 1945), 2,6-diiodo-4-aminophenol (Woollett et al., 1937) and 2,4,6-triiodoanisole (Drew and Sturtevant, 1939) were prepared by methods cited in the respective references. All other chemicals were of reagent grade.

Enzyme and Coenzyme. Approximately 80% pure HRP was obtained from Boehringer Co. The enzyme (10 mg in 1 mL) in 50% $(NH_4)_2SO_4$ was dialyzed overnight at 4 °C against 3 L of 0.05 M sodium phosphate buffer (pH 7.4). Its concentration was then determined by its absorbancy at 403 nm ($\epsilon = 9 \times 10^4$ cm^{-1} M^{-1}). The peroxidase activity of the enzyme was measured by the increase of its optical density at 485 nm in the presence of *p*-phenylenediamine and H_2O_2 (Lück, 1963). Yeast alcohol dehydrogenase and NADH were purchased from Boehringer and Oriental Yeast Co., respectively. Horse heart cytochrome *c* was obtained from Sigma Chemical Co. Ferrocytochromei5ifcizas prepared by the reduction of ferricytochrome *c*izith sodium dithionite followed by anaerobic dialysis. Superoxide dismutase was prepared from bovine erythrocytes and assayed in terms of its ability to inhibit the superoxide-dependent reduction of cytochrome *c* by xanthine oxidase (McCord and Fridovich, 1969).

Assay Conditions. The standard incubation mixture for the assay of NADH oxidation consisted of 3.3×10^{-4} M H_2O_2, 1×10^{-4} M NADH, 1×10^{-7} M HRP, 1.67×10^{-5} M phenolic compound, and 0.067 M sodium phosphate buffer (pH 7.4) in a total volume of 3 mL. Unless otherwise noted, all reactions were carried out 25 °C. The concentrations of components are listed in the legends or in the text. The oxidation of NADH was monitored at 340 nm, using a molar extinction coefficient of 6.2×10^3 M^{-1}. The oxidation and reduction of cytochrome *c* were monitored at 550 nm using the molar extinction coefficients given by Massey (1959). Oxygen consumption was recorded on a Yanagimoto oxygenometer.

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[1] Abbreviations used are: HRP, horseradish peroxidase; NADH, reduced nicotinamide adenine dinucleotide; T_4, thyroxine.

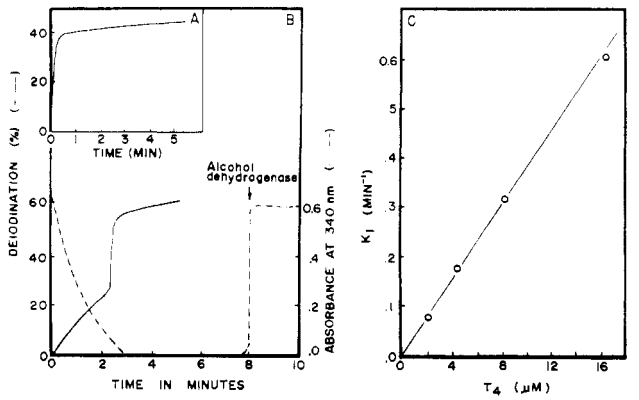


FIGURE 1: Deiodination of thyroxine by HRP-H_2O_2 (A) and thyroxine-mediated oxidation of NADH by HRP-H_2O_2 (B). The reaction mixture containing 0.067 M sodium phosphate (pH 7.4), 3.3×10^{-4} M H_2O_2, 1×10^{-4} M NADH, and 1.67×10^{-5} M thyroxine with a tracer of L-[3′,5′-^{131}I]thyroxine in 2.9 mL of water was preincubated at 25 °C for 5 min, and then the reaction was initiated by the addition of 1×10^{-7} M HRP in 0.1 mL of H_2O at time zero. In the case of experiment A, NADH was omitted. After completing NADH oxidation, the reaction mixture was made alkaline with NaOH and 0.1 mL of ethanol, and 10 μL of alcohol dehydrogenase (80 units) was added at the time indicated by an arrow. Apparent first-order rate constants (K_1) were plotted vs. initial concentration of thyroxine (C).

Photometric assays were performed with a Cary Model 17. Degradation of L-[3′,5′-^{131}I]thyroxine was assayed as previously described (Nakano et al., 1971).

Results

Thyroxine as Catalyst of NADH Oxidation. The time courses of thyroxine deiodination and NADH oxidation by the HRP-H_2O_2 system (standard incubation mixture) are shown in Figure 1A,B.

When most of the NADH was oxidized, the thyroxine was also largely deiodinated. Alternatively, during the time when the oxidation of NADH was in progress, thyroxine deiodination was slight. The addition of alcohol dehydrogenase to the spent reaction mixture yielded nearly all of the added NADH, establishing that NAD^+ was the only oxidized compound produced from NADH. Omission of NADH from the system caused a rapid deiodination of the thyroxine (but this never exceeded over 50%). Under our experimental conditions, little if any NADH was oxidized in the absence of thyroxine.

With fixed concentrations of reactants NADH (1×10^{-4} M), H_2O_2 (3.3×10^{-4} M), and enzyme (1×10^{-7} M) (except for thyroxine), until 65% NADH was oxidized, the oxidation of NADH occurred at a pseudo-first-order rate with respect to NADH and was of zero order with respect to thyroxine concentration (Figure 1C). The rate can be calculated from

$$-\left(\frac{d[NADH]}{dt}\right) = K_0[T_4][NADH] = K_1[NADH] \quad (1)$$

where $K_0[T_4] = K_1$ is the apparent first-order rate constant in which $[T_4]$ represents the initial concentration of thyroxine ($K_0 = 3.9 \times 10^4$ M^{-1} min^{-1}). The high efficiency of the NADH oxidation at low substoichiometric levels of thyroxine indicates that this hormone acts as catalyst in the oxidation of NADH in the HRP-H_2O_2 system.

Relationship between Structure and Potency of Catalysts in NADH Oxidation. Thyroxine analogues were tested as possible catalysts on the NADH oxidation in the HRP-H_2O_2 system (standard incubation mixture).

NADH oxidations measured with all of the compounds were apparent first-order reactions and their potencies were expressed by the corresponding first-order rate constants (Table I). Of the compounds tested, 3,5,3′,5′-tetraiodothyropropionic acid was the most potent. Thyroxine analogues with two iodine atoms on the β-phenyl ring (thyroxine and 3′,5′-diiodothyronine) were more effective than those with only one iodine on the β-phenyl ring (3,5,3′-triiodothyronine and 3,5,3′-triiodothyroacetic acid) and 3,5-diiodothyronine without an iodine on the β-phenyl ring. Hence, iodine on the β-phenyl ring contributes to potency. Unexpectedly, thyronine, without iodine on both α- and β-phenyl rings, also manifested high potency, but it catalyzed NADH oxidation only during the first minute.

TABLE I: Apparent First-Order Rate Constants in Thyroxine Analogue-Mediated Oxidation of NADH by Peroxidase and H_2O_2.

Substrate[a]	K_1
Thyroxine	0.61
3′,5′-Diiodothyronine	0.36
3,5,3′-Triiodothyronine	0.27
3,5-Diiodothyronine	0.21
3,5,3′,5′-Tetraiodothyropropionic acid	1.80
3,5,3′-Triiodothyroacetic acid	0.29
3,5-Diiodo-3′-isopropylthyronine	0.00
Thyroxine methyl ester	0.15
Tyrosine	0.00
3,5-Diiodotyrosine	0.00
2,6-Diiodo-4-aminophenol	1.80
2,4,6-Triiodophenol	0.28
2,6-Diiodohydroquinone	0.92
2,6-Diiodo-4-nitrophenol	0.03
2,4,6-Triiodoanisole	0.01

[a] All the compounds were tested at 1.67×10^{-5} M.

The results presented here are quite similar to those obtained during the oxidation of epinephrine rather than NADH in a comparable system (Klebanoff, 1959a). Under our experimental conditions, 3′-isopropyl-3,5-diiodothyronine, known to produce a powerful hormonal activity in vivo (Greenberg et al., 1963), had no demonstrable effect upon NADH oxidation.

In thyroxine and in related molecules, the ionization and electron density of the OH group could be expected to be affected by adjacent iodine atoms and para substituents, respectively. Furthermore, this OH group donates one electron easily if an adequate acceptor is present (Wynn and Gibbs, 1963). However, structural complexity in these compounds makes it difficult to clarify the above problems. The relationship between electron-releasing activity of OH and para substituents would be in part clarified if less complex analogues such as the 2,6-diiodophenols could be used as catalysts in our system.

All of the phenolic compounds that activate NADH oxidation in the standard reaction mixture acted as catalysts which, as with thyroxine and related compounds, enhanced the first-order rate of NADH oxidation. The 2,6-diiodophenolic compounds (*p*-NH_2, *p*-OH and *p*-I substituents) were found to be potent catalysts, whereas other phenolic compounds without iodine substituents (hydroquinone, *p*-aminophenol and *tert*-butylhydroquinone) had less, if any, activity. Plots of the rate constants of phenolic compounds and Hammett σ values of the para substituents yielded a straight line with $\rho = -0.9$. Under the same experimental conditions, 4-nitro-2,6-diiodo-

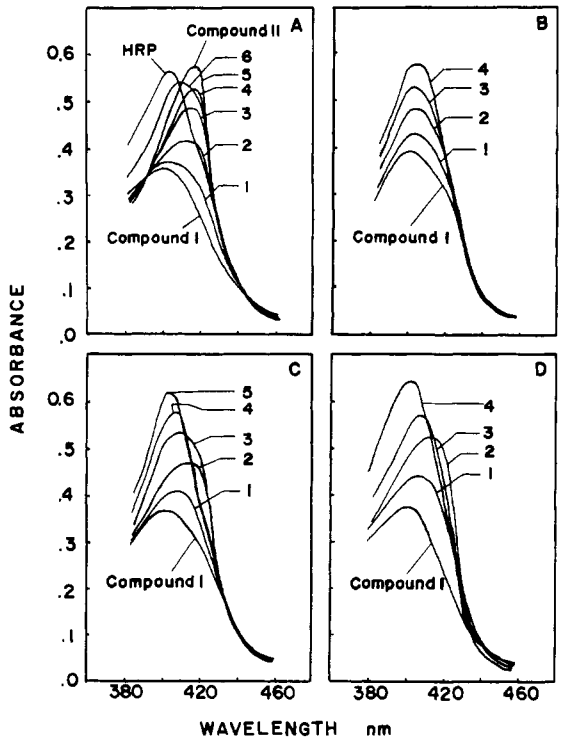


FIGURE 2: Spectrometric observation of compound I (ferriperoxidase-H_2O_2 complex) in the presence of NADH (A), I^- (B), thyroxine (C), or NADH plus I^- (D). In all cases, HRP (7×10^{-6} M) was initially converted to compound I by an equimolar addition of H_2O_2 in 0.067 M sodium phosphate buffer (pH 7.4) to a final volume of 3 mL. Both the spectrum of the original HRP and that of compound I were recorded. Beginning 90 s after the H_2O_2 addition, the stepwise addition of an electron donor was started. The volume of each addition was 10 μL and the additions were made at 90-s intervals. Spectrum 1 was recorded just after the first addition, spectrum 2, just after the second, etc. In A, NADH (1.4 μM) was added stepwise; in B, I^- (1.4 μM) was added stepwise; in C, thyroxine (1.4 μM) was added stepwise; in D, a mixture of NADH (1.4 μM) and I^- (1.4 μM) was added stepwise.

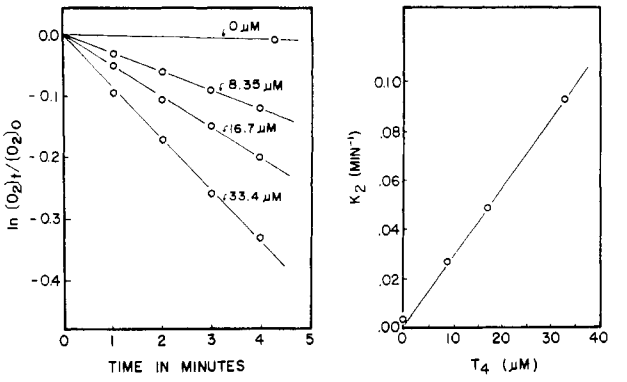


FIGURE 3: Oxygen consumption during thyroxine-mediated oxidation of NADH by HRP and H_2O_2 (A). The reaction mixture (3 mL) consisted of 0.02 M sodium phosphate buffer (pH 7.4), 1×10^{-4} M H_2O_2, 2×10^{-4} M NADH, 4×10^{-8} M HRP, and various concentrations of thyroxine as indicated in the figure. All components, except for thyroxine, were preincubated for 1 min at 25 °C and the reaction was initiated by the addition of thyroxine. $[O_2]_0$ and $[O_2]_t$ represent O_2 concentration at zero time and that at t minutes after the addition of thyroxine, respectively. Apparent first-order rate constants (K_2) obtained from the slopes in Figure 3A were plotted vs. initial concentrations of thyroxine (B).

phenol ($K_{1(min^{-1})} < 0.03$) which contains p-NO_2 (known to have strong electron-attracting ability) and the methyl ether of 2,4,6-triiodophenol ($K_{1(min^{-1})} < 0.01$) had no significant activity. These results clearly indicate that OH is the important group in catalytic oxidation of NADH and that its activity is greatly influenced by either electron-releasing or -attracting ability of para substituents.

Spectroscopic Observation of Ferriperoxidase-Thyroxine (Iodine, NADH)-H_2O_2 Interaction. If the HRP-H_2O_2 complex, compound I (absorption maximum at 403 nm) accepts one electron from thyroxine, NADH, or a product of thyroxine (I^-), it would be converted to compound II (absorption maximum at 418 nm) which, in turn, could be converted to free enzyme by one-electron reduction (Chance, 1952). Hence, we recorded the spectrum of compound I while it was gradually converted to free HRP or to a related species by the stepwise addition of electron donor.

When NADH was the electron donor, compound I (prepared by the addition of H_2O_2 in equimolar concentrations to 7×10^{-6} M HRP) was first converted to compound II and only then to free HRP (Figure 2A). On the other hand, a gradual conversion of the spectrum of compound I to one resembling that of free HRP (maximum at 403 nm) was observed following the addition of I^- as the electron donor (Figure 2B). This duplicates the finding reported by Björksten (1970), who proposed that the newly formed compound is a HRP-iodine complex.

When a combination of I^- and NADH was used as electron donors, the spectral change in compound I (Figure 2D) was quite different from that observed with NADH alone or I^- alone. Thus, the former did not show the typical peak absorbance at 418 nm of compound II or the 403-nm peak of the HRP-I_2 complex. A similar spectral change was also observed with thyroxine (Figure 2C), suggesting that both the HRP-I_2 complex and compound II could be produced by the interaction of compound I and thyroxine.

The relative rate of conversion of compound I to compound II was measured by photometry following the single addition of an electron donor (1.67 μM) to a cuvette which contained compound I prepared by the addition of H_2O_2 in an equimolar concentration to 6.4×10^{-6} M HRP and observed during the initial 15 s at 410 nm (isosbestic point of HRP-compound II) (Yonetani, 1966). Under these conditions, the rate obtained with thyroxine was ten times faster than that recorded with NADH. The addition of a small quantity of thyroxine obviously accelerated the rate of conversion of compound I to compound II by NADH.

Requirements of O_2 for the Oxidation of NADH. When thyroxine was added to the HRP (0.04 μM)-NADH (200 μM)-H_2O_2 (100 μM) system, oxygen consumption increased significantly and its rates obeyed first-order kinetics with respect to O_2 concentration and zero order with respect to thyroxine concentration (Figure 3). The rate equation can be written as

$$-\left(\frac{d[O_2]}{dt}\right) = K_0'[T_4][O_2] = K_2[O_2] \qquad (2)$$

where $K_0'[T_4] = K_2$ is the apparent first-order rate constant and $[T_4]$ is initial concentration of thyroxine. The K_0' obtained under these conditions was 2.8×10^3 M^{-1} min^{-1}.

The relationship between O_2 consumption and NADH oxidation in the presence of thyroxine is shown in Table II. Under these conditions, 0.83 mol of O_2 is required for the oxidation of 1 mol of NADH.

Generation of $O_2^{-}\cdot$. To demonstrate the generation of a superoxide anion ($O_2^{-}\cdot$) in the course of NADH oxidation in the HRP-H_2O_2 system, ferricytochrome c was added as a trapper of $O_2^{-}\cdot$. Its reduction was monitored by the increase of absorbance of the media at 550 nm in the presence or ab-

TABLE II: Relationship between Oxygen Consumption and NADH Oxidation.[a]

Thyroxine Concn (μM)	Time (min)	NADH Oxidized (μM)	O_2 Consumed (μM)
0.835	0.5	3.9 (1.0)[b]	3.35 (0.86)[b]
	1.0	7.8 (1.0)	6.40 (0.82)
1.67	0.5	7.8 (1.0)	6.45 (0.83)
	1.0	14.8 (1.0)	12.9 (0.87)

[a] The components of the reaction mixture were the same as those of the complete system described in the legend of Figure 5, except for the concentration of thyroxine. [b] Numbers in parentheses represent the values which are relative to μM NADH oxidized, taken as unity. [c] With the system excluding thyroxine, neither O_2 consumption nor NADH oxidation was observed during the time cited.

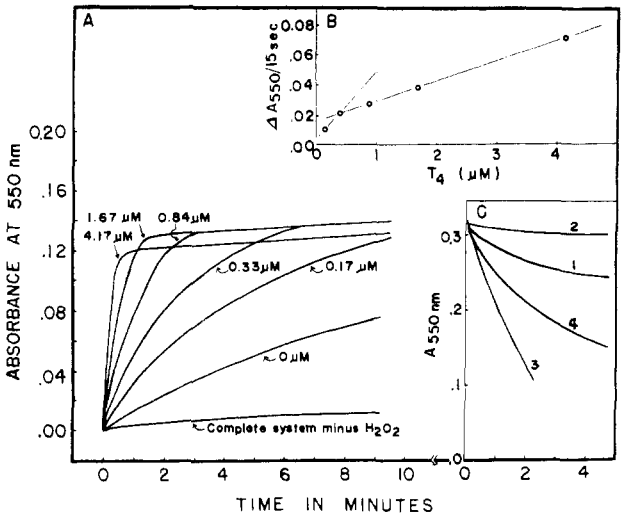


FIGURE 4: Reduction of ferricytochrome c (A,B) and oxidation of ferrocytochrome c (C) during thyroxine-mediated oxidation of NADH by HRP and H_2O_2. The reaction conditions of experiment A were described in the text. Absorbance changes in the reduction of ferricytochrome c during the initial 15 s were plotted vs. initial concentrations of thyroxine (B). In the oxidation of ferrocytochrome c (C), the complete reaction system (1) was the same as that in experiment A, except that 8.35×10^{-7} M thyroxine and 1×10^{-5} M ferrocytochrome c were used. The other reaction systems were: (2) complete systems minus thyroxine, (3) complete system minus NADH, (4) complete system plus 1×10^{-7} M superoxide dismutase.

sence of superoxide dismutase. The reaction mixture used for these experiments contained 0.02 M sodium phosphate buffer (pH 7.4), 10 μM H_2O_2, 200 μM NADH, 0.2 μM HRP, 20 μM ferricytochrome c, and various concentrations of thyroxine indicated in Figure 4A.

Under the specified conditions, thyroxine enhanced the initial rate of reduction of ferricytochrome c in the HRP-H_2O_2-NADH system. Increasing the level of thyroxine increased the initial rate of reduction of ferricytochrome c, but a linear relationship between them was demonstrable only at low concentration of thyroxine (Figure 4A,B).

On the other hand, ferrocytochrome c was, indeed, oxidized to ferricytochrome c in our HRP-H_2O_2-NADH system. The rate of its oxidation was accelerated by thyroxine or by the omission of NADH from the system (Figure 4C), identical with those reported by others (Klebanoff, 1960). In the same figure, it can be seen that superoxide dismutase enhanced the oxidation of ferrocytochrome c in the HRP-H_2O_2-NADH

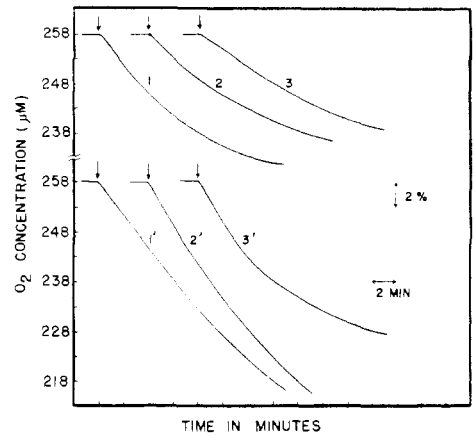


FIGURE 5: Effects of superoxide dismutase and/or ferricytochrome c on the oxygen consumption during thyroxine-mediated oxidation of NADH by HRP and H_2O_2. The complete system (1) consisted of 0.02 M phosphate buffer (pH 7.4), 1×10^{-5} M H_2O_2, 2×10^{-4} M NADH, 2×10^{-7} M HRP, and 8.35×10^{-7} M thyroxine in 3 mL. The other reaction systems were: (2) complete system plus 2×10^{-5} M cytochrome c, (3) complete system plus 4×10^{-5} M cytochrome c, (1′) complete system plus 1×10^{-7} M superoxide dismutase, (2′) complete system plus 2×10^{-5} M cytochrome c plus 1×10^{-7} M superoxide dismutase, (3′) complete system plus 4×10^{-5} M cytochrome c plus 1×10^{-7} M superoxide dismutase.

system, probably by preventing the reduction of oxidized cytochrome c by the $O_2^{-}\cdot$ generated in the experiment. Since oxidoreduction of cytochrome occurred in this system, the rate of reduction of cytochrome c (Figure 4A,B) should be corrected for the rate of oxidation of ferrocytochrome c.

In a system which consisted of 1×10^{-5} M H_2O_2, 2×10^{-4} M NADH, 2×10^{-7} M HRP, 8.35×10^{-7} M thyroxine, 2×10^{-5} M ferricytochrome c, and 0.02 M sodium phosphate buffer (pH 7.4), superoxide dismutase at 1×10^{-8} M and 1×10^{-7} M produced a 63% and a 100% inhibition of the reduction of ferricytochrome c, respectively. There was only 10% and 45% inhibition with heat-inactivated dismutase at the same concentrations. The enzyme had no effect on NADH oxidation, indicating that $O_2^{-}\cdot$ did not participate in the oxidation of NADH.

Figure 5 depicts the inhibition by ferricytochrome c of O_2 consumption and its prevention by superoxide dismutase. Ferricytochrome c at 2×10^{-5} M slightly inhibited and at 4×10^{-5} M halved the initial rate of O_2 uptake. Superoxide dismutase at 1×10^{-7} M completely prevented the inhibition of oxygen consumption by cytochrome c, and clearly accelerated the rate of O_2 uptake in the late stages of the reaction, both in the presence and absence of cytochrome c at 2×10^{-5} M. Doubling the concentration of the ferricytochrome c reduced the effect of superoxide dismutase on late-stage oxygen consumption. In view of the inhibition of O_2 uptake by ferricytochrome c at 4×10^{-5} M, one can conclude that under these conditions about 54% of the total flux of electrons from NADH to oxygen goes via the univalent route.

Discussion

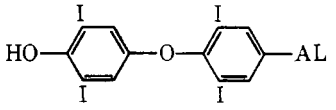
On the basis of kinetic studies of the rate of NADH consumption in the presence of various concentrations of thyroxine, it is clear that thyroxine acts as a catalyst for NADH oxidation in the HRP-H_2O_2 system.

Diiodophenols with electron-releasing groups at their para position act, as do thyroxine and its analogues, as powerful mediators or catalysts of NADH oxidation. The close relations between Hammett σ values of the para substituents of diiodophenols and their NADH-oxidizing properties provide proof

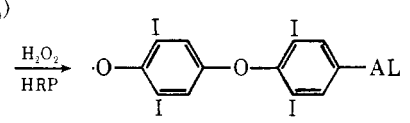
that not only the electron densities of the OH groups in the iodinated phenols but also those in thyroxine analogues play an important role in NADH oxidation in the HRP-H_2O_2 system.

The spectral changes of the HRP complex produced by stoichiometrical doses of the peroxidase and H_2O_2 (1:1) and by the addition of thyroxine suggest that thyroxine acts as an electron donor to compounds I and II even though iodine produced during the reaction interferes with clear evidence of the formation of compound II. NADH is more easily demonstrable as an electron donor than is thyroxine, but the rate of the electron-releasing activity of NADH is much slower than that of thyroxine.

From this point of view, in the initial step of thyroxine-mediated NADH oxidation by HRP and H_2O_2 under the usual assay conditions oxidized thyroxine is formed (eq 3) and, in



(T_4)



(T_4·) (3)

$$T_4\cdot + NADH \longrightarrow T_4 + NAD\cdot \quad (4)$$

turn, rapidly oxidizes NADH (eq 4). The resulting NAD· reacts rapidly with O_2 to produce $O_2^-\cdot$ and NAD^+ (eq 5).

$$NAD\cdot + O_2 \rightarrow NAD^+ + O_2^-\cdot \quad (5)$$

Willson (1970) has reported a second-order rate constant of $1.9 \times 10^9\ M^{-1}\ s^{-1}$ for this reaction. Since 2 mol of $O_2^-\cdot$ undergo spontaneous dismutation, the system produces O_2 and H_2O_2 (eq 6).

$$O_2^-\cdot + O_2^-\cdot \xrightarrow{2\,H^+} O_2 + H_2O_2 \quad (6)$$

This process is catalyzed by superoxide dismutase. The spontaneous reaction is relatively slow (Behar et al., 1970), while the enzyme-catalyzed reaction is very rapid with a second-order rate constant of $1.7 \times 10^9\ M^{-1}\ s^{-1}$ (Forman and Fridovich, 1973). If the system included saturation amounts of ferricytochrome *c*, most of the $O_2^-\cdot$ generated is trapped by ferricytochrome *c* (eq 7).

$$\text{ferricytochrome } c + O_2^-\cdot \rightarrow \text{ ferrocytochrome } c + O_2 \quad (7)$$

Molecular oxygen generated by this reaction should be utilized for the oxidation of NAD·. If superoxide dismutase is also present, it competes with ferricytochrome *c* for $O_2^-\cdot$. It thus tends to overcome the effect of cytochrome *c* on the oxygen consumption, yielding H_2O_2 in the system. Also, some of the generated $O_2^-\cdot$ can be used for the conversion of HRP to compound III (eq 8).

$$HRP + O_2^-\cdot \rightleftharpoons \text{compound III} \quad (8)$$

Since compound III is less reactive than the other forms of HRP (Yamazaki and Piette, 1963), O_2^- inhibits peroxidase activity and superoxide dismutase prevents or reverses this inhibition by competing with HRP for the available $O_2^-\cdot$.

This series of four reactions (eq 5-8) could account for (1) the observed stoichiometry involved in O_2 consumption and NADH oxidation, (2) the inhibition of O_2 consumption by ferricytochrome *c* and its prevention by superoxide dismutase, and (3) the prevention of inactivation of HRP by superoxide dismutase (monitored by oxygen uptake).

Even though about 50% of NADH oxidation via univalent electron transfer could be accounted for by the inhibition of O_2 consumption by ferricytochrome *c*, this percentage is unduly low because a portion of $O_2^-\cdot$ could be effectively trapped by free HRP (eq 8) (Yamazaki and Piette, 1963).

The NADH oxidation by our system closely resembles the aerobic oxidation of dihydroxy fumarate catalyzed by peroxidase in the presence of H_2O_2 (Yamazaki and Piette, 1963). However, this is quite different from the NADH oxidation by peroxidase in the presence of Mn^{2+}, O_2, and certain phenolic compounds in that the latter requires 0.5 mol of O_2 for the oxidation of 1 mol of NADH (Akazawa and Conn, 1958; Williams-Ashman et al., 1959; Klebanoff, 1959b).

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A Spectroscopic and Kinetic Investigation of Anion Binding to Ascorbate Oxidase†

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ABSTRACT: The binding of azide, fluoride, and cyanide to ascorbate oxidase has been investigated in detail. Both azide and fluoride inhibit the enzyme competitively with respect to ascorbic acid and noncompetitively with respect to oxygen. Cyanide inhibition is much more complex and also results in inactivation of the enzyme. The binding of azide and fluoride to the resting enzyme is partially competitive. Fluoride binds more strongly to the resting enzyme, while azide binds more strongly to the functioning enzyme. It is proposed that both azide and fluoride bind to type 2 copper and that this copper is also part of an ascorbate binding site. It seems likely that type 2 copper is a reductant binding site in all of the "blue" oxidases. This proposal is used to explain the effect of fluoride on these enzymes and also to suggest a mechanism for the internal electron transfer which is necessary for the reduction of oxygen to water.

Ascorbate oxidase (EC 1.10.3.3, L-ascorbate:O_2 oxidoreductase), along with laccase and ceruloplasmin, comprise a group of enzymes commonly known as "blue" copper oxidases. All members of this group have many similar properties and the state of copper in these enzymes has been reviewed (Malmström et al., 1975).

All of these enzymes contain three types of copper. Fungal laccase, containing a total of four copper atoms, has been studied in detail with respect to the enzyme properties associated with the copper (Malkin et al., 1968, 1969; Brändén and Reinhammar, 1975). It has been suggested, based on total copper content and electron paramagnetic resonance and visible spectral properties, that both ascorbate oxidase and ceruloplasmin contain twice as much of each type of copper as laccase and that these two enzymes possess two "laccase-type" active sites of four copper atoms each (Strothkamp and Dawson, 1974; Mondovi et al., 1975). In agreement with this type of arrangement is the presence of two, identical, half-molecular-weight subunits in both ascorbate oxidase (Strothkamp and Dawson, 1974) and ceruloplasmin (Mukasa et al., 1968; Freeman and Daniel, 1973). If this proposal is correct, the results concerning the copper in laccase can also be applied to the other two enzymes. It should be noted that other suggestions have also been made concerning the stoichiometry of copper (Deinum et al., 1974; Veldsema and VanGelder, 1973; Deinum and Vänngård, 1973) and quaternary structure (Rydén, 1972; Simons and Bearn, 1969) of ascorbate oxidase and ceruloplasmin.

The catalytic mechanism of the "blue" oxidases is very complex. Both ascorbate oxidase and laccase are reduced via several one-electron transfers from substrate, as shown by the detection of free radicals formed from the substrate during the reaction (Yamazaki and Piette, 1961; Broman et al., 1963). The reduced form of the enzyme then reduces molecular oxygen to water by the addition of four electrons, probably via two, two-electron transfers (Malmström, 1970; Andréasson et al., 1973a). That all three types of copper in these enzymes are involved in the catalytic mechanism has been shown for laccase (Malkin et al., 1968, 1969), and some kind of internal electron transfer must take place (Andréasson et al., 1973b).

A number of reports on laccase and ceruloplasmin have confirmed the binding of various anions (Malkin et al., 1968; Kasper, 1968; Brändén et al., 1973; Byers et al., 1973; Herve et al., 1975) with subsequent changes in spectral properties and loss of catalytic activity, but much of the data is conflicting. A recent report has indicated an interaction between ascorbate oxidase and azide (Mondovi et al., 1975). It was felt that a detailed study of the anion binding properties of ascorbate oxidase could reveal much about the catalytic mechanism of this enzyme, as well as confirm additional similarities between all of the "blue" oxidases.

Experimental Section

Materials. Ascorbate oxidase was obtained from green zucchini squash (*Cucurbita pepo medullosa*) as previously described (Lee and Dawson, 1973), and was homogeneous by polyacrylamide gel electrophoresis at pH 9.5, according to the procedure of Davis (1964). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, Mo.). All reagents were of the highest purity available and all aqueous solutions were prepared just prior to use using deionized water.

Methods. Protein concentration was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as standard. Copper determinations were performed according to Stark and Dawson (1958). Enzymatic activity was measured at 25 °C in a 1.5-mL reaction volume using a Clark oxygen electrode on a Gilson Model KM Oxygraph, under concentration and buffer conditions previously described (Dawson and Magee, 1957).

Spectral titrations were carried out using a Cary 118C recording spectrophotometer equipped with a thermostated cell holder maintained at 25 ± 0.3 °C. Both sample and blank were identical solutions of oxidized enzyme in 0.20 M phosphate-citrate, pH 5.6. Each addition of ligand to the sample was matched by an identical amount of deionized water added to the blank to maintain equal protein concentrations. Absorbance values were corrected for dilution during the titrations.

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