Re-evaluation of the Kinetics of Lactate Dehydrogenase-catalyzed Chain Oxidation of Nicotinamide Adenine Dinucleotide by Superoxide Radicals in the Presence of Ethylenediaminetetraacetate*

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The chain oxidation of lactate dehydrogenase-bound NADH initiated by superoxide radicals and propagated by oxygen was studied with pulse radiolysis. The kinetic parameters were re-evaluated in a system with carefully purified reagents (water and other chemicals) and in the presence of EDTA.

The rate constant for the oxidation of the enzyme-bound NADH by \( O_2^- \) is calculated from the observed pseudo-first order disappearance of NADH and the chain length (molecules of NADH oxidized per \( O_2^- \) anion generated in the pulse). It is \( (1.0 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{s}^{-1} \), consistent within a 13-fold variation in lactate dehydrogenase NADH complex concentration and with varying chain length up to 6.1. Based on experiments with varying pH values from 4.5 to 9.0, the rate constant for oxidation of enzyme-bound NADH by \( O_2 \) is estimated to be \( 2.0 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \).

In an aqueous solution superoxide and hydroperoxyl radicals are in equilibrium with a \( pK = 4.8 \) (1).

\[ \text{HO}_2^- \rightleftharpoons H^+ + O_2^- \quad K_{1,-1} = 1.6 \times 10^{-5} \text{ M} \quad (1, -1) \]

Our earlier reports (2–4) have shown that the two radical forms \( \text{HO}_2 \) and \( O_2^- \) react with lactate dehydrogenase-bound NADH at different rates, and that the nucleotide radical produced in either Reaction 3 or Reaction 4 reacts in turn with molecular oxygen (Reaction 5) to generate another superoxide radical. The chain oxidation mechanism was proposed as follows:

\[ \text{LDH} + \text{NADH} \rightleftharpoons \text{LDH} \cdot \text{NADH} \quad (2, -2) \]
\[ \text{LDH} \cdot \text{NADH} + \text{O}_2^- \rightarrow \text{LDH} \cdot \text{NAD}^- + \text{HO}_2^- \quad (3) \]
\[ \text{LDH} \cdot \text{NADH} + \text{HO}_2 \rightarrow \text{LDH} \cdot \text{NAD}^- + \text{H}_2\text{O}_2 \quad (4) \]
\[ \text{LDH} \cdot \text{NAD}^- + \text{O}_2 \rightarrow \text{LDH} \cdot \text{NAD}^+ + \text{O}_2^\cdot \quad (5) \]
\[ \text{LDH} \cdot \text{NAD}^+ \rightleftharpoons \text{LDH} + \text{NAD}^+ \quad (6, -6) \]

Under the experimental conditions in this and earlier studies (4), the termination of the chain reaction followed pseudo-first order kinetics, indicating that the disproportionation of superoxide radicals did not contribute significantly toward its destruction. Consequently a general termination step is proposed, Reaction 7,

\[ X + \text{O}_2^- \rightarrow \text{product(s)} \quad (7) \]

where \( X \) represents an unknown scavenger for \( \text{HO}_2^-/O_2^- \).

Most of the experiments in this study were carried out in the neutral pH range, in which the ionized form of the oxygen radical is the predominant species; therefore Reaction 3 is of primary interest in this study. In the discussion that follows, for simplicity, the sum of the radicals (\( O_2^- + \text{HO}_2 \)) are referred to as superoxide radicals.

The reason for our reinvestigation of the kinetics of this chain mechanism (Reactions 2 to 6) is that although we did observe good pseudo-first order kinetics for the disappearance of NADH over a 4-fold LDH·NADH concentration range (4), at that time we were unable to establish a consistent value of the chain propagation rate constant (\( k_p \) or \( k_s \)).

The decay of the superoxide radicals is very sensitive to impurities, particularly in the physiological and alkaline pH range. Despite great efforts by several research groups good second order kinetics for the decay has not yet been established for this pH range; most reports describe mixed kinetics of first and second order (1, 5–15).

These difficulties have now been traced to catalytic impurities which we have been able to eliminate to a large degree by the addition of EDTA. Klug et al. (16) have found in pulse radiolysis studies that EDTA does not react with superoxide radicals. The present report is a reassessment of the kinetic parameters of the chain mechanism in the presence of EDTA.

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1*The abbreviation used in equations is: LDH, lactate dehydrogenase.
Our results obtained under varying conditions now give consistent values of $k_\phi$.

The experimental procedures and equipment used have been described in detail in our earlier publication (4). Water used in this study was triply distilled from a 2 m sodium formate and alkaline permanganate and then subsequently redistilled from 1 m EDTA in an all quartz distilling apparatus. The monosodium and disodium phosphate salts were added to sodium formate recrystallized three times from this purified water.

The DEER experiments were carried out in the absence of EDTA. De Graaff was regulated by varying energy input of the pulse. The number of active sites was evaluated by the Holbrook method (18).

The main objective of this study was the re-evaluation of $k_\phi$ at the physiological pH, hence the bulk of the data were determined at pH 7.35 and 7.5 (Table I). Under these conditions the rate of disappearance of the superoxide radical by Reaction 7 instead of $\mathrm{H}_2\mathrm{O}_2$ in Reaction 3 is equal to its rate of regeneration in this system, and the equation reduces to

$$-\frac{d[\mathrm{O}_2^-]}{dt} = k_\phi[\mathrm{LDH} - \mathrm{NADH}] + k_4[X][\mathrm{O}_2^-] - k_{\mathrm{LDH} - \mathrm{NADH}}[\mathrm{O}_2^-]$$

(II)

Since $k_\phi \gg k_4$ (4), the rate of disappearance of $\mathrm{O}_2^-$ in Reaction 3 is equal to its rate of regeneration in this system, and the equation reduces to

$$-\frac{d[\mathrm{O}_2^-]}{dt} = k_\phi[X][\mathrm{O}_2^-]$$

(III)

**TABLE I**

| Run No. | $[O_2^-]/[H_2O]$ | Δ[NADH] | $\psi$ | $k_4$ | $k_{\mathrm{LDH} - \mathrm{NADH}}$ |
|---------|------------------|---------|--------|-------|------------------|
| 1       | 0.10             | 0.24    | 2.40   | 0.70  | 17.1             |
| 2       | 0.27             | 0.46    | 1.70   | 0.87  | 17.1             |
| 3       | 0.47             | 0.58    | 1.93   | 1.31  | 17.1             |
| 4       | 0.10             | 0.58    | 5.80   | 1.02  | 46.6             |
| 5       | 0.23             | 1.20    | 5.22   | 0.80  | 46.6             |
| 6       | 0.37             | 1.75    | 4.73   | 1.15  | 46.6             |
| 7       | 1.00             | 3.91    | 3.21   | 2.12  | 46.6             |
| 8       | 0.15             | 0.92    | 6.13   | 0.99  | 51.9             |
| 9       | 0.25             | 1.20    | 4.80   | 1.24  | 51.9             |
| 10      | 0.45             | 1.39    | 3.09   | 1.41  | 51.9             |
| 11      | 0.93             | 2.38    | 2.56   | 1.90  | 51.9             |
| 12      | 1.75             | 4.44    | 2.34   | 2.21  | 51.9             |
| 13      | 2.50             | 4.90    | 1.96   | 2.13  | 51.9             |
| 14      | 3.70             | 5.12    | 1.38   | 3.33  | 51.9             |
| 15      | 1.68             | 1.79    | 1.07   | 0.42  | 3.97             |
| 16      | 1.77             | 2.61    | 1.47   | 0.25  | 3.97             |
| 17      | 1.77             | 2.59    | 1.46   | 0.25  | 3.97             |
| 18      | 1.82             | 2.61    | 1.43   | 0.25  | 3.97             |
Kinetics of Chain Oxidation of LDH·NADH by O₂⁻ and HO₂

where X is an unknown compound or certain protein functional group (or groups) in the system which destroys the superoxide radical in the termination step, Reaction 7. Hence the only reaction by which the superoxide radical concentration is diminished during the chain reaction is by the termination step (Reaction 7) described by Equation II, which upon integration yields

\[ [O_2^-] = [O_2^-]_0 e^{-k_7't} \]  

(III)

The rate of disappearance of NADH by Reaction 3 is given by Equation IV.

\[-d[LDH\cdot NADH]/dt = k_3[O_2^-][LDH\cdot NADH] \]  

(IV)

Substitution of Equation III into Equation IV yields

\[-d[LDH\cdot NADH]/dt = k_3[LDH\cdot NADH][O_2^-]e^{-k_7't} \]  

(V)

The total change \( \Delta [NADH] \) observed for the chain reaction is obtained upon integration of Equation V between the corresponding time limits \( t = 0 \) and \( t = \infty \):

\[ \Delta [NADH] = k_3[O_2^-]_0[LDH\cdot NADH]/e^{-k_7't} dt \]  

(VI)

or

\[ k_4 = \frac{k_3[X][\Delta [NADH] /[LDH\cdot NADH]_0]_0}{[LDH\cdot NADH]_0} \]  

(VII)

Under present experimental conditions \( t = 0 \) is for all practical purposes the end of the electron pulse, which generates a known quantity of superoxide radical \([O_2^-]_0\). \( k_4[X] \) is manifested by the experimentally observed pseudo-first order rate constant \( k_{cat} \) for the disappearance of NADH and \( \Delta [NADH] \) is the total change in NADH concentration for the given run. Pseudo-first order conditions were maintained throughout a given experiment and the quantity \([LDH\cdot NADH]_0\) can be considered as constant since the total change in NADH concentration represented only a small fraction of the total amount present, and under such conditions rapid regeneration of the complex at the expense of the excess NADH pool keeps the \( LDH\cdot NADH \) concentration constant due to the favorable conditions of the equilibrium constant \( K_{LDH\cdot NADH} = 3.9 \times 10^{-7} \) M (19).

A second order rate constant \( k_{cat} \) can be defined as \( k_4[X]/[LDH\cdot NADH]_0 \) or \( k_{cat}/[LDH\cdot NADH]_0 \). Equation VII shows that this calculated constant multiplied by the chain length (\( \nu = \Delta [NADH]/[O_2^-]_0 \)) when \( \nu \gg 1 \) gives \( k_4 \). These correlations are illustrated in Table I. The \( k_4 \) values (Column 8) determined from runs with chain length up to 6.1 and a 13-fold concentration range of the \( LDH\cdot NADH \) complex are in agreement within experimental error.

While in an earlier study (4) we found, under similar pulse conditions but in the absence of EDTA, a maximum chain length of 18, the present results show only an optimal chain length of 6.18 (Run 8) at pH 7.35. Apparently in the earlier report (4) catalytic impurities affected both the chain length and the kinetics of this system.

The anchor points in the pH curve in Fig. 1 are the average values of 14 runs at pH 7.35 and 22 runs at pH 7.50 from Table I. The ratio of \( O_2^-/HO_2 \) at these two pH values are 358/1 and 505/1, respectively. Thus the contribution of Reaction 4 to the overall rate of disappearance of NADH was of the order of 6 and 4%, respectively. As is apparent from the data in Table I, statistically, one cannot differentiate between the two series of experiments. The average value of all the runs listed in Table I computed by the standard deviation method is \( k_4 = (1.0 \pm 0.2) \times 10^4 \) M⁻¹ s⁻¹.

In the pH range where Reaction 4 contributes to the disappearance of \( LDH\cdot NADH \), the second order rate constant \( k_{cat} \) has to be expressed in terms of the equilibrium constant (1, 4):

\[ k_{cat} = k_3[X]/[LDH\cdot NADH]_0 \frac{K_{LDH\cdot NADH}}{K_{LDH\cdot NADH} + [O_2^-]/[HO_2^-]} \]  

(VIII)

Based on the dissociation constant of superoxide radicals and the calculated rate constants at various pH values, an estimated second order rate constant for Reaction 4 was obtained: \( k_4 = 2.0 \times 10^4 \) M⁻¹ s⁻¹. A theoretical curve computed with Equation VIII is shown as a solid line in Fig. 1. It fits the experimental points between pH 4.7 and 9.0. The value for \( k_4 \) should be taken only as an approximation because the change in \( K_{LDH\cdot NADH} \) with pH is not well established and has not been taken into consideration in estimating \( k_4 \).

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