Kinetic Study by Pulse Radiolysis of the Lactate Dehydrogenase-catalyzed Chain Oxidation of Nicotinamide Adenine Dinucleotide by HO$_2$ and O$_2^-$ Radicals*

(Received for publication, June 19, 1974)

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SUMMARY

The lactate dehydrogenase-catalyzed chain oxidation of NADH (LDH-NADH) by the superoxide radicals, HO$_2$ and O$_2^-$, has been studied with pulse radiolysis in the pH range between 4.5 and 9.0. The rate constants for the oxidation of the LDH-NADH by HO$_2$ and O$_2^-$ determined at 23° are $1.2 \times 10^6$ M$^{-1}$ s$^{-1}$ and $3.6 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively. The latter represents an activation of over 1000-fold by the enzyme. A chain reaction mechanism consistent with the results from these kinetic studies has been proposed.

Earlier reports (1, 2) have shown that lactate dehydrogenase (EC 1.1.1.27) catalyzes the superoxide radical-induced chain oxidation of reduced nicotinamide adenine dinucleotide in the presence of molecular oxygen. The superoxide radical necessary for the induction of the chain reaction can be generated either by $^{60}$Co $\gamma$ rays or by the xanthine-O$_2$-xanthine oxidase system (3).

The purpose of the present investigation is to determine the rates of interaction between the lactate dehydrogenase-bound NADH and the superoxide radicals (which refer to HO$_2$ and O$_2^-$ in equilibrium). Because of the fast reaction rates, the technique of pulse radiolysis was chosen to generate superoxide radicals in an oxygenated sodium formate solution. It has been established that although formate protects lactate dehydrogenase against radiation damages (4), it does not affect the catalytic activity of the enzyme on NADH oxidation by pyruvate (1). The molecular oxygen plays a multiple role in this system; it serves as a precursor for the HO$_2$ and O$_2^-$ radicals, it protects the enzyme against reductive attack by the hydrated electron and atomic hydrogen (Reactions 1 and 2), and it also acts as a chain carrier (Reaction 9).

Based on the results from various studies (4–6), the over-all mechanism for the formation of HO$_2$ and O$_2^-$ radicals in this system can be presented in the following reactions:

\[
\begin{align*}
\text{H}_2\text{O} & \quad \text{e}^- \quad \text{O}_2 \quad \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 & \quad k_3 \quad 1.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \quad \text{HO}_2 \\
\text{H}_2\text{O}_2 & \quad k_4 \quad 2.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O} + \text{CO}_2 \\
\text{CO}_2^- & \quad k_5 \quad 4.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \quad \text{O}_2^- + \text{CO}_2 \\
\text{OH} + \text{H}_2\text{O}_2 & \quad k_6 \quad 2.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O} + \text{CO}_2^- \\
\text{H}_2 \quad k_7 \quad 4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H} + \text{O}_2^- \\
\text{H} + \text{O}_2^- & \quad k_8 \quad 1.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 \\
\text{OH} + \text{H}_2\text{O}_2 & \quad k_9 \quad 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 + \text{H}_2\text{O} \\
\text{H}_2\text{O}_2 + \text{H}_2\text{O} & \quad k_{10} \quad 4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 \\
\text{O}_2^- + \text{H}_2\text{O}_2 & \quad k_{11} \quad 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{OH} + \text{O}_2^- & \quad k_{12} \quad 1.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 \\
\text{H}_2\text{O} + \text{O}_2^- & \quad k_{13} \quad 4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 \\
\text{H} + \text{O}_2^- & \quad k_{14} \quad 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 \\
\text{OH} + \text{O}_2^- & \quad k_{15} \quad 1.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 \\
\text{O}_2^- + \text{H}_2\text{O}_2 & \quad k_{16} \quad 4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \quad \text{H}_2\text{O}_2 \\
\end{align*}
\]

The numerical values in parentheses in Equation 1 represent the G values, the number of molecules formed or transformed per 100 e. v. of energy dissipated in the system. Most conveniently, G values are computed from calibrations with the ferrous dosimeter, G(Fe$^{3+}$) = 15.5 (7). In the above mechanism, the G value for the total number of superoxide radicals formed is $G(\text{HO}_2 + \text{O}_2^-) = 6.05$. In the presence of high formate concentrations (0.1 M and higher), this G value is slightly higher on account of the spur scavenging. In the present investigation, the experimentally determined $G(\text{HO}_2 + \text{O}_2^-) = 6.45$. With this G value, a ferrous dosimeter calibration curve, and the monitored energy input per pulse, one can calculate the concentration of superoxide radicals produced in the irradiated sample (5).

METHODS

Pig heart lactate dehydrogenase obtained from Boehringer Mannheim Corp. (Catalog No. 15372) was dialyzed against 0.01 M phosphate buffer, pH 7.5, at 4°. After the elimination of small amounts of insoluble materials by centrifugation, the enzyme concentration was determined at 280 nm, using the molar extinction coefficient of $1.97 \times 10^6$ M$^{-1}$ cm$^{-1}$ (8). A determination of the total number of active sites was carried out using the method of Holbrook (9). Samples of the enzyme solutions used for radiation studies were prepared by diluting the stock solution with sodium formate solution (the final concentration of sodium formate was 0.1 M). The pH of various samples was adjusted by the addition of either sodium hydroxide or phosphoric acid.

* Research was performed, in part, under the auspices of the United States Atomic Energy Commission. This paper is a part of a series on enzyme-catalyzed free radical reactions with nicotinamide adenine nucleotides.
FIG. 1. Effect of enzyme concentration (LDH-NADH = total number of active sites) upon the pseudo-first order oxidation rate of NADH by superoxide anion (O$_2^-_r$). The reaction mixture contained 50 mM of phosphate, 0.25 mM O$_2$, 0.1 M sodium formate, and varying amounts of NADH and LDH-NADH (pH 8.1; 23°C). Aliquots of 4 ml of this solution were irradiated with a single 10-μs pulse of 1.9 m.e.v. electrons which generated 1.2 μM O$_2^-$ in a given sample.

| Curve | [M] | [μM] | $k_{obs}$ | $k_{l}

NADH and NAD$^+$ (Sigma Chemical Co.) were used without further purification. All of the other chemicals used were of reagent grade. Solutions were prepared in triply distilled water.

The pulsing electron generator (2 m.e.v. Van de Graaff), optics, and monitoring equipment have been described in detail earlier (10).

RESULTS

The rates for the lactate dehydrogenase-catalyzed oxidation of NADH by HO$_2$ and O$_2^-$ were studied with pulse radiolysis in oxygenated sodium formate solutions. The LDH-NADH solutions were always prepared by diluting a stock solution of freshly dialyzed enzyme, which had been titrated for the total number of active sites with the method of Holbrook (9).

Following an electron pulse, the reaction was monitored by observing the disappearance of NADH in the spectral region between 340 and 380 nm. Because the total light path of the optical setup was 0.1 cm, it was often necessary, due to the high absorbance, to measure the changes in NADH concentration at a wavelength other than the spectral maximum at 340 nm. For a particular set of experiments, the pulse length as well as the energy input per pulse was kept constant. On the average, the variation in the energy input per pulse and hence the initial concentration of superoxide radicals generated was of the order of 4 to 5%.

Because preliminary pulse experiments revealed that the observed rate of the disappearance of NADH followed pseudo-first order kinetics in the presence of lactate dehydrogenase, a number of solutions with varying amounts of enzyme were studied at pH 8.1 in order to determine the second order rate constant for Reaction 8. The experimental conditions and results are given in Fig. 1. The good agreement between the second order rate constants computed from the $k_{obs}$ values at different enzyme concentrations, supports the assumption that under the given experimental conditions, all of the active sites on the enzyme were occupied by NADH.

The study of the pH effect upon the rates of oxidation of the LDH-NADH complex by HO$_2$ and O$_2^-$ radicals is shown in Fig. 2. The experiments were limited on the acidic side (below pH 4.5) by the instability of NADH, the denaturation of the enzyme, and the decreasing signal to noise ratio. Similarly, on the alkaline side, the signal to noise ratio made accurate determinations impractical beyond pH 9.0. The results show that the $k_{obs}$ did not change significantly between pH 9 and pH 7, whereas it increased sharply below pH 7.

In the same series of experiments, the total decrease in absorbance within 20 s after the electron pulse was determined and converted to the amount of NADH oxidized. Because the amount of superoxide radical generated by the pulse could be calculated from the energy input, the ratio of the number of molecules of NADH oxidized per superoxide radical (i.e., the chain length) for each experimental point can be determined. As can be seen in Fig. 3, the chain length has a maximum at pH 7.2 and it drops off almost symmetrically with increasing either acidity or alkalinity.

DISCUSSION

The qualitative aspects of the lactate dehydrogenase-catalyzed chain oxidation of NADH by superoxide radicals had been established and discussed in detail in earlier reports (1, 2). The present study was aimed at the establishment of some of the

1 The abbreviation used is; LDH-NADH, lactate dehydrogenase-nicotinamide adenine dinucleotide complex.
kinetic parameters of the reactions which describe this chain mechanism:

\[
\begin{align*}
LDH + NADH & \rightleftharpoons K_{LDH-NADH} \times 10^{-6} \rightarrow LDH-NADH \quad (6, -6) \\
LDH-NADH + H_2O_2 & \rightarrow k_7 \times 1.2 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \rightarrow LDH-NADH + H_2O_2 \quad (7) \\
LDH-NADH + O_2^- + H_2O_2 & \rightarrow k_8 \times 3.6 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \rightarrow LDH-NADH + H_2O_2 \quad (8) \\
LDH-NADH + O_2 & \rightarrow k_9 \times 10^8 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \rightarrow LDH-NADH + O_2^- \quad (9) \\
LDH-NADH & \rightarrow K_{LDH-NADH} \times 10^{-6} \rightarrow LDH + NAD^+ \quad (10, -10)
\end{align*}
\]

Lactate dehydrogenase binds NADH much more strongly than NADH. In this study, based on the over-all magnitude of the dissociation constant for the LDH-NADH complex (11, 12), NADH was always present in excess to saturate all of the active sites of the enzyme. Hence, the effective concentration of the complex LDH-NADH in Reactions 7 and 8 is equal to the experimentally determined total number of active sites. This assumption was supported by the observation of strict pseudo-first order kinetics over a 4-fold concentration of LDH-NADH.

The observed pseudo-first order decay of NADH (Fig. 1) over several half-lives indicates that the effective complex concentration of LDH-NADH is maintained at a constant level over many chain lengths (compare with Fig. 3), the reason being that the rates controlling its replenishment (Reactions 6, 9 and 10), from the excess (0.1 mm) pool of unbound NADH are more rapid than those of its consumption (Reactions 8 and 9). Hence, it may be assumed that one of the essential factors for the chain reaction is the difference in magnitude between the dissociation constants of LDH-NADH and LDH-NADH.

The change in the pseudo-first order constant \(k_{obs}\) with pH is shown in Fig. 2. The plateau region between pH 7 and 9 represents Reaction 8 with little or no contribution from Reaction 7. The second order rate constant calculated was \(k_8 = 3.6 \pm 0.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}\). As the pH is decreased, the equilibrium \((\text{HO}_2^- = \text{O}_2^- + \text{H}_2\text{O})\) is shifted to the left and hence in favor of Reaction 7. The relative increase in \(k_{obs}\) below pH 6, suggests that \(\text{HO}_2^-\) reacts more rapidly with the LDH-NADH complex than \(\text{O}_2^-\). Because the rate constant for Reaction 7 could not be determined in isolation, that from the corresponding plateau region (see Fig. 2, dashed line between pH 1 and 3), an approximate value for \(k_7 = 1.2 \times 10^8 \text{ M}^{-1} \text{s}^{-1}\) was computed by Equation II from the experimental points along the upper slope between pH 4.5 and 5.5. Equation II was derived from the two controlling reactions, 7 and 8, and the dissociation constant of \(\text{HO}_2^-\) (Reaction 3, -3); \(k_{II}\) describes the overall rate of disappearance of LDH-NADH where \([A]\) is the LDH-NADH concentration and \(X = K_{\text{HO}_2^-}/[\text{H}^+]\):

\[
k_{II} = [A] \left[ \frac{k_7 + k_8 X}{1 + X} \right]
\]

As is apparent in Fig. 2, the value for \(k_7\) gives a fairly good fit between the computed line and the experimental points. The dashed part of the curve is the extrapolated pH profile in the acid region if both the enzyme and NADH were stable and active.

Although the concentration of the unbound NADH in the reaction mixture was about 6 times as high as that of the LDH-NADH complex, the interaction between superoxide radicals and unbound NADH did not contribute significantly to the rate of oxidation. Land and Swallow (13) estimated that \(k_{II} \ll 27 \text{ M}^{-1} \text{s}^{-1}\).

The difference by a factor of 108 between \(k_8\) and \(k_{II}\) dramatically illustrates the catalytic effect of lactate dehydrogenase in this oxidation step.

Reaction 9, in which the enzyme-bound radical LDH-NADH reacts rapidly with molecular oxygen, is the chain propagating step. The assumption that Reaction 9 is very rapid, is based on the reported rate constant (13, 14) for Reaction 12 of the unbound radical with molecular oxygen:

\[
\begin{align*}
NAD^+ + O_2^- + \text{LDH-NADH} \quad & \rightarrow k_{12} \times 1.9 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \rightarrow NAD^+ + H_2O_2 \quad (12)
\end{align*}
\]

and on experimental observations made in the present study. An attempt to search for the absorbance of the LDH-NAD as an intermediate species in the presence of molecular oxygen after the pulse has not been successful. The signal was of the order of the noise level, which indicated an extremely low steady state concentration and a very rapid Reaction 9. A similar experiment carried out in the absence of molecular oxygen showed that the enzyme stabilized the free radical by many orders of magnitude. The disappearance of the free radical (Reactions 13 and 14) could be observed for several minutes at 405 nm by yielding the dimer as postulated by Land and Swallow (15) in Reaction 14.

\[
\begin{align*}
LDH-NAD^+ & \rightarrow k_{13} \times 10^9 \text{ M}^{-1} \text{s}^{-1} \rightarrow \text{LDH-NAD}^+ \quad (13)
\end{align*}
\]

Unpublished results.
This observation confirms that Reaction 9 is fast and precludes the possibility of dissociation in Reaction 13 to be followed by Reaction 12 in the chain mechanism. Hence, Reaction 9 leads to the formation of the LDH-NAD\(^+\) complex which dissociates (Reaction 10) and the vacant site is replenished with another molecule of NADH (Reaction 6). Thus, the concentration of LDH-NADH remains constant throughout several cycles as indicated by the pseudo-first order kinetics in Fig. 1.

The true nature of the termination step has not yet been established due to experimental difficulties. In the absence of appropriate reactants, the superoxide radicals decay by disproportionation. Various studies \((10, 16-18)\) have determined the following reaction constants:

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{H}_2\text{O} & \xrightarrow{k_{15}} 7.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \quad \text{H}_3\text{O}^+ + \text{O}_2^- \\
\text{H}_2\text{O}_2 + \text{O}_2^- & \xrightarrow{k_{16}} 8.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \quad \text{H}_3\text{O}^+ + \text{O}_2^- + \text{OH}^- \\
\text{O}_2^- + 2\text{H}^+ & \xrightarrow{k_{17}} 2 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \quad \text{H}_3\text{O}^+ + \text{O}_2^- + 2\text{OH}^- 
\end{align*}
\]

\[(15)-(17)\]

The same studies have also found that when traces of impurities were present in the systems, the superoxide radicals decayed with pseudo-first order kinetics, indicating interaction between the radicals and the impurities. Hence, one can assume a \textit{pri}ori that the addition of an enzyme to a solution will introduce “impurities” as scavengers for the superoxide radicals. These scavengers could be some reactive groups on the protein, which do not have a detectable effect on the enzyme activity. A study on the influence of lactate dehydrogenase on the decay of superoxide radicals in the absence of NADH did show some acceleration of the decay rate. Although this slight increase in the rate of disappearance of superoxide radicals was negligible in determining the rate constants of Reactions 7 and 8, it possibly could contribute to the termination of the chain reaction along with Reactions 15, 16, and 17.

For an approximate estimation of the pH effect on the decay of superoxide radicals, one can calculate the over-all rate of decay as a function of H\(^+\) concentration in the absence of scavengers by Equation III:

\[
k_{111} = \frac{k_{15} + k_{16}X + k_{17}X^2}{(1 + X)^2}
\]

where \(X = K_{\text{HO}}^*/\text{H}^+\)

\[
pH \quad k_{111}, \text{ M}^{-1} \text{s}^{-1} \\
4.0 \quad 1.07 \times 10^7 \\
5.0 \quad 2.02 \times 10^7 \\
6.0 \quad 4.71 \times 10^6 \\
7.0 \quad 5.25 \times 10^5 \\
8.0 \quad 5.32 \times 10^4 \\
9.0 \quad 5.41 \times 10^3
\]

It is apparent that with decreasing pH the rate of over-all decay of superoxide radicals increases rapidly and thus tends to shorten the chain length. On the other hand, a decrease in pH is accompanied by an increase in the HO\(_2\)-O\(_2^-\) ratio and thereby increases the rate of over-all oxidation as indicated in Fig. 2, and therefore the latter factor tends to lengthen the chain. Consequently, when the chain length was plotted against pH, an optimal chain length of 18 was found at pH 7.2.

An earlier study \((1)\) had shown that one of the factors controlling the chain length is the dose rate, because it determines the ratio of LDH-NADH:O\(_2^-\). As expected, the chain length under pulse radiolysis conditions is not as high and competition by Reactions 15, 16, and 17 for Reactions 7 and 8 is more efficient.

Termination of the chain reaction by a radical-radical interaction (Reaction 18) can be ruled out in this system, because Reaction 12 is very fast, and the ratio of O\(_2\):O\(_2^-\) is of the order of 500.

\[
\text{LDH-NAD} + \text{O}_2^- + 2\text{H}^+ \longrightarrow \text{LDH-NAD}^+ + \text{H}_2\text{O}_2 (18)
\]

Although the termination step is still not precisely defined, the results derived from these kinetic studies have provided definitive support for the chain mechanism of oxidation of LDH-NADH by superoxide radicals presented in Reactions 6, 7, 8, 9, and 10.

REFERENCES

1. BIELSKI, B. H. J., AND CHAN, P. C. (1973) Arch. Biochem. Biophys. 159, 873-879
2. CHAN, P. C., AND BIELSKI, B. H. J. (1974) J. Biol. Chem. 249, 1317-1320
3. FRIDOVICH, I. (1970) J. Biol. Chem. 245, 4033-4037
4. BIELSKI, B. H. J., AND GEHICKI, J. M. (1970) in \textit{Advances in Radiation Chemistry} (Burtan, M., and Magee, J. L., eds) Vol. 2, pp. 177-279, Wiley-Interscience, New York
5. BIELSKI, B. H. J., AND ALLEN, A. O. (1969) J. Radiat. Phys. Chem. 1, 153-163
6. ANBAR, M., AND NETA, P. (1967) J. Appl. Radiat. Isotopes 18, 490-523
7. ALLEN, A. O. (1961) \textit{The Radiation Chemistry of Water and Aqueous Solutions}, p. 35, Van Nostrand, Princeton, N. J.
8. PISCHE, A., MCKAY, R. H., STOLENBACH, F., CANN, R. D., AND KAPLAN, N. O. (1964) \textit{J. Biol. Chem.} 239, 1753-1761
9. HOLBOER, J. J. (1966) \textit{Rinchem. Z.} 344, 141-152
10. BIELSKI, B. H. J., AND SCHWARZ, H. A. (1968) J. Phys. Chem. 72, 3836-3841
11. SCHWERT, G. W., MILLER, B. R., AND PEANSKI, R. J. (1967) \textit{J. Biol. Chem.} 242, 3245-3252
12. HECK, H. yA. (1969) \textit{J. Biol. Chem.} 244, 4375-4381
13. LAND, E. J., AND SWALLOW, A. J. (1971) \textit{Biochem. Biophys. Acta} 234, 34-42
14. WILSON, R. L. (1970) \textit{Chem. Commun.} 1005
15. LAND, E. J., AND SWALLOW, A. J. (1968) \textit{Biochem. Biophys. Acta} 162, 327-337
16. CZAPSKI, G., AND BIELSKI, B. H. J. (1969) \textit{J. Phys. Chem.} 67, 2180-2184
17. RABANI, J., AND NIELSEN, S. O. (1969) \textit{J. Phys. Chem.} 73, 3736-3744
18. BEHAR, D., CZAPSKI, G., RABANI, J., DORFMAN, L. M., AND SCHWARZ, H. A. (1970) \textit{J. Phys. Chem.} 74, 2309-2313
Kinetic study by pulse radiolysis of the lactate dehydrogenase-catalyzed chain oxidation of nicotinamide adenine dinucleotide by HO2 and O2-RADICALS.
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J. Biol. Chem. 1975, 250:318-321.

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