SPECTRAL CHARACTERISTICS OF LACTATE OXYGENASE IN AEROBIC AND ANAEROBIC REACTIONS *

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1. Introduction

Lactate oxygenase from *Mycobacterium phlei* has been shown to be an FMN-containing flavoprotein which catalyzes the incorporation of molecular oxygen into L-lactate, forming acetate as product [1, 2]. However, there has been no quantitative investigations to follow out the detailed reaction mechanism of this catalytic process since Beinert and Sands [3] noted the transient appearance of a long wavelength absorbing intermediate on addition of substrate. Recent studies from this laboratory [4] have demonstrated that the enzyme-bound $FMNH_2$ and pyruvate corresponding to the dehydrogenated product are formed when a substrate level amount of the enzyme is incubated anaerobically with an equivalent amount of L-lactate, but the oxygenated product, acetate is not produced by admitting air into the reduced enzyme. This finding is quite different from that found previously with salicylate hydroxylase in which the enzyme-bound $FADH_2$ is an essential electron donor for the hydroxylation reaction [5, 6]. From these results it seems that the flavin of lactate oxygenase does not function in manner similar to that proposed for salicylate hydroxylase. Therefore, at present, there is less evidence for the participation of flavin in the lactate oxygenase reaction than in the salicylate hydroxylase reaction.

In this paper, we report the stopped flow analysis of the spectral behavior of flavin during aerobic and anaerobic reactions of lactate oxygenase.

2. Materials and methods

Crystalline lactate oxygenase from *Mycobacterium phlei* was prepared as reported previously [7]. The crystals were dissolved in 50 mM potassium phosphate buffer pH 7.0, and dialyzed against the same buffer to remove contaminating ammonium sulfate completely. The substrate, L-lithium lactate was obtained from Miles Laboratories, Inc., Elkhart, Indiana. The flow experiments were performed using a temperature-controlled flow system [8] which was essentially the same as that designed by Chance and Legallais [9]. The semitangential 4-jet mixing chamber used in the flow system was a modification of that used by Millikan [10]. In anaerobic experiments the syringes of the flow apparatus were bubbled and equilibrated with nitrogen.

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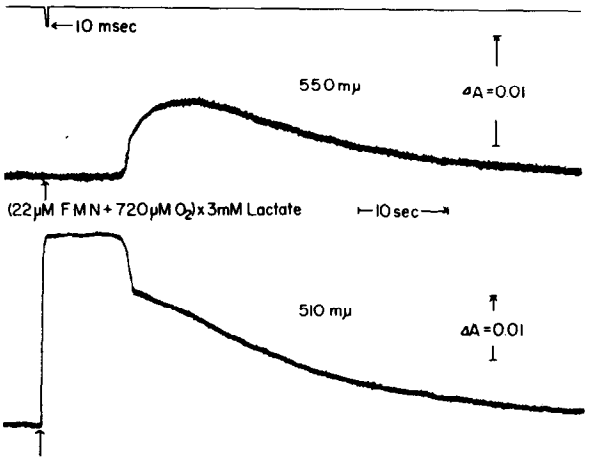

Fig. 1. Changes in absorbance at 550 and 510 mμ on mixing 22 μM lactate oxygenase (molarity with respect to the enzyme-bound FMN), 3 mM L-lactate and 720 μM O_2 (the concentrations are those after mixing). The reactions were carried out in 50 mM potassium phosphate buffer, pH 7.0 at 25°. The traces before mixing indicate the level of the reduced enzyme.

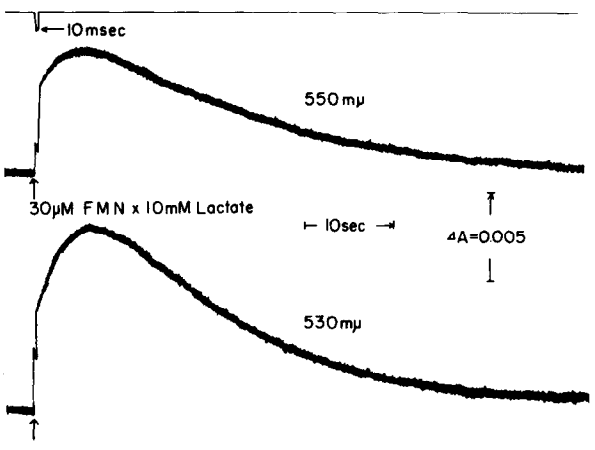

Fig. 3. Changes in absorbance at 550 and 530 mμ on mixing 30 μM lactate oxygenase and 10 mM L-lactate under anaerobic conditions. The reactions were carried out in 50 mM potassium phosphate buffer, pH 7.0 at 25°. The traces before mixing indicate the level of the reduced enzyme.

3. Results and discussion

Fig. 1 shows the changes in absorbance at 550 and 510 mμ in an experiment designed to determine the visible spectrum of lactate oxygenase produced by the aerobic addition of L-lactate. Under aerobic conditions, the steady state appeared after the flow stopped and the rate of oxygen utilization was calculated to be 3.5 sec^{-1}. As the oxygen concentration was depleted, the absorption at 550 mμ increased simultaneously and reached the maximum in 16.5 sec. The absorption then disappeared much more

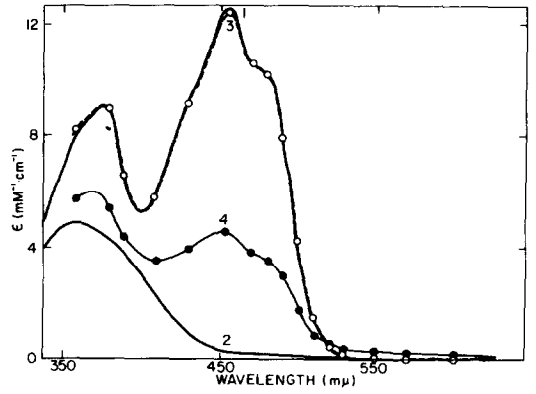

Fig. 2. Spectra obtained at various times in the experiment described in fig. 1. Curves 1 and 2 were obtained with a Cary 14 recording spectrophotometer. Curve 1, oxidized enzyme; Curve 2, enzyme reduced with L-lactate anaerobically; Curve 3, spectrum obtained at the steady-state level; Curve 4, spectrum obtained when the absorbance value at 550 mμ was maximal.

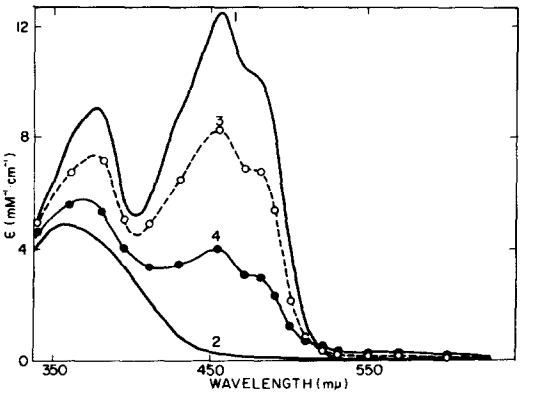

Fig. 4. Spectra obtained at various times in the experiment described in fig. 3. Curve 1, oxidized enzyme; Curve 2, enzyme reduced with L-lactate anaerobically; Curve 3, spectrum obtained immediately after the flow stopped; Curve 4, spectrum obtained when the absorbance value at 550 mμ was maximal.

slowly with a decay half-time of 9.5 sec. These observations indicate that the 550-mμ absorption appears transiently under anaerobic conditions. At 510 mμ, a rapid decrease in absorbance proceeded with a parallel increase in absorbance at 550 mμ. Absorbance changes following mixing of the enzyme and L-lactate were measured at various wavelengths. Fig. 2 shows the plots of the extinction coefficient of the enzyme thus obtained at each wavelength. It is evident from curve 3 (broken line) that the enzyme is highly oxidized in the steady state and no spectroscopically detectable species exist in the steady state which differ from those in the fully oxidized state. When the absorbance value at 550 mμ attained the maximum, the spectrum as indicated in curve 4 was obtained. It is of particular interest that a species with a weak absorption band at a long wavelength appeared.

When L-lactate was anaerobically mixed with the enzyme, the absorptions at 550 and 530 mμ increased simultaneously. It is seen in fig. 3 that there is a distinct biphasic increase in absorption. The half-time of the absorbance change occurring in the fast phase of the reaction in the presence of 10 mM L-lactate was 15 msec. The absorbance then fell very slowly until the enzyme was completely reduced. When the absorbance changes occurring in the fast phase of the reaction were plotted at each wavelength, the absorption spectrum as indicated in curve 3 of fig. 4 was obtained. When the enzyme was mixed with a final concentration of 10 mM L-lactate, the pseudo-first-order rate constant for the fast phase was 46 sec^{-1}, which was considerably greater than the turnover number in the over-all aerobic reaction (9.5 sec^{-1}). Therefore, if this species is common to both aerobic and anaerobic reactions, it does not participate in a rate-limiting step in the over-all aerobic reaction. As shown in curve 4 of fig. 4, the absorption spectrum obtained when the absorbance value at 550 mμ was maximal was very similar to that observed in curve 4 of fig. 2. The development of a broad, low absorption band at a long wavelength was again apparent. These results strongly suggest that this species might be the transient intermediate in the process of pyruvate formation under anaerobic conditions.

Acknowledgements

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