OSCILLATORY OXIDATIONS OF REDUCED PYRIDINE NUCLEOTIDE BY PEROXIDASE

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Received November 15, 1965

It is widely recognized that periodic phenomena are common in biological systems. The nature of the oscillator driving such phenomena has engaged the attention of many physiologists and biochemists. Recently clear damped oscillations of the reduced pyridine nucleotide level have been found in Yeast cells (Ghosh and Chance, 1964; Chance, Estabrook and Ghosh, 1964; Hommes, 1964 a, 1964 b) and in cell-free extracts (Chance, Hess and Betz, 1964; Chance, Schoener and Elsaesser, 1964). It was shown that the waveform of oscillations depends upon the level of metabolite intermediate in the glycolytic system (Chance, Schoener and Elsaesser, 1964). This communication describes the observation of periodic reactions catalyzed by peroxidase in the presence of reduced pyridine nucleotide under aerobic conditions.

The detailed mechanism of the aerobic oxidation of reduced pyridine nucleotide catalyzed by peroxidase has been reported (Yokota and Yamazaki, 1965). Fig. 1 shows a slightly modified mechanism. The previous paper describes three characteristic phenomena of the reaction, which might cause the periodic reactions. 1. Irregular dependency of the oxidation velocity upon the concentration of reduced pyridine nucleotide. No appreciable aerobic oxidation is observed at low levels of the reduced pyridine nucleotide. The oxidation velocity increases exponentially with the concentration of reduced

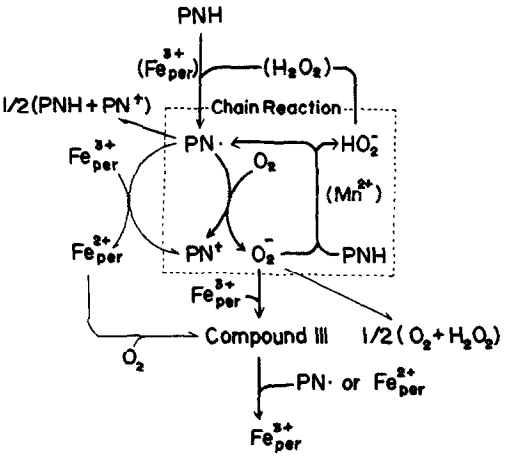

Fig. 1. Possible mechanism of aerobic oxidation of the reduced pyridine nucleotide. This mechanism also suggests the possible paths of formation and decay of Compound III during the reaction.

pyridine nucleotide. 2. Accumulation of peroxidase Compound III during the reaction. Compound III, however, is not a typical active intermediate. In the presence of excess peroxidase the rapid oxidation of NADH only occurs when most of the enzyme has been converted to Compound III. Compound III, then, seems to be an inactive intermediate of the enzyme trapping an active intermediate, probably the perhydroxyl radical. 3. Disappearance of Compound III after oxygen has been consumed in the presence of excess reduced pyridine nucleotide. Under these conditions peroxidase exists as a mixture of the ferric and ferrous forms. The ratio between the ferric and ferrous forms of the enzyme depends mainly on the pH and the concentration of reduced pyridine nucleotide (Yokota and Yamazaki, 1965).

As can be seen in Fig. 2, addition of glucose-6-phosphate dehyrogenase to a solution containing NADP, Mn^{2+}, glucose-6-phosphate and peroxidase results in a transient reduction of NADP, followed by a few cyclic responses of oxidation and reduction until all of the oxygen is consumed. As the NADP is reduced at a constant rate during the reaction in Fig. 2, it can be seen that a remarkably rapid oxidation of NADH suddenly occurs at a certain reac-

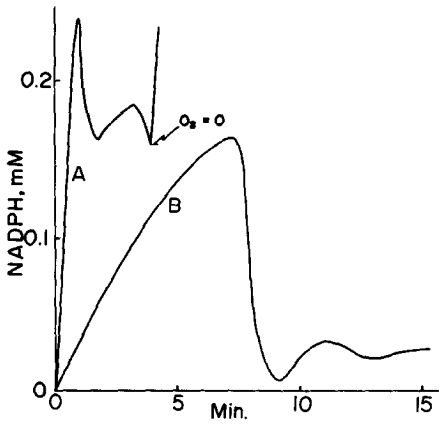

Fig. 2. Kinetics of NADP reduction in the presence of glucose-6-phosphate dehydrogenase and peroxidase. 4 mM glucose-6-phosphate, 1 mM $NADP^+$, 10 µM horse-radish peroxidase, 0.05 M acetate (pH = 5.2), 25°. A ; 0.33 mM Mn^{2+}, 20 µg glucose-6-phosphate dehydrogenase per 3 ml. B ; 1 mM Mn^{2+}, 2 µg glucose-6-phosphate dehydrogenase per 3 ml. In the presence of high dehydrogenase very sharp reduction of NADP can be observed immediately after oxygen has disappeared. The crystalline peroxidase was prepared from wild horse-radish and the ratio of E_{403} to E_{278} for this enzyme was 3.4.

tion time (one minute in the case, A) which corresponds to the termination of Compound III accumulation. The reaction may produce hydrogen peroxide, which is again used to keep the reaction going further until a low level of NADPH is reached wherein the efficiency of the chain reaction in Fig. 1 is no longer sufficient for the reaction to proceed. A part of Compound III seems to go back to the ferric state and the NADPH oxidation slows down. It is very difficult to get highly reproducible reactions when using a combination of pyridine nucleotide reductase and horse-radish peroxidase. Most pyridine nucleotide reductases have optimum activity at a slightly alkaline pH, while horse-radish peroxidase catalyzes the aerobic oxidation of reduced pyridine nucleotide most effectively at an acidic pH.

More stable periodic reactions can be observed in the oxidation of NADH when oxygen is supplied continuously by bubbling air diluted with nitrogen into the solution. Addition of NADH to an aerobic solution of peroxidase results in a rapid appearance of Compound III as is illustrated in Fig. 3. The rate of NADH oxidation increases suddenly when the formation of Compound

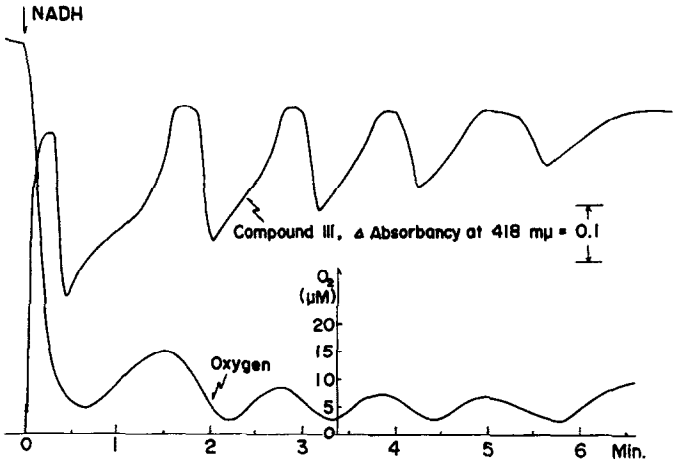

Fig. 3. Oscillation of oxygen concentration in the presence of NADH and peroxidase when nitrogen-oxygen mixed gas (24 : 1) is bubbled into the reaction solution at constant rate (10 ml per minute). Reaction starts with the addition of NADH (final concentration = 0.8 mM). Increase in absorbancy at 418 mμ is due to the formation of Compound III (Yokota and Yamazaki, 1965) and recorded by a Hitachi recording spectrophotometer. Oxygen concentration was followed simultaneously by using a sputtered platinum electrode which was kindly supplied by professor M. Mochizuki in this Institute. This type of oscillation stops when NADH is consumed and repeats again on the further addition of NADH. Total reaction volume in the wide cell (light path = 1 cm) is 6 ml. 10 μM horse-radish peroxidase, 0.1 M acetate (pH 5.12), 25°.

III is completed as was reported previously (Yokota and Yamazaki, 1965). The rate of concomitant consumption of oxygen overcomes that of the oxygen supply and the oxygen concentration of the solution decreases, followed by the decomposition of Compound III. As oxygen is supplied continuously the accumulation of Compound III is again observed and the oxdation reaction repeats itself until almost all of the NADH is oxidized. It may be suggested, from this experiment, that a more sustained periodicity of the reaction would be expected provided the level of NADH remained constant, as it does for example, in the presence of certain NAD reductase systems. The use of a peroxidase which has a high activity for aerobic oxidation of NADH at neutral pH's might solve this problem.

The formation and decay of Compound III seems to be an essential mechanism responsible for the periodicity of the reaction which is observed

in Fig. 3. The formation of Compound III is mainly due to the reaction of peroxidase with the perhydroxyl radical which causes the chain reaction of the NADH oxidation. It is probably also formed in the reduction of peroxidase by the NAD radical, the product of which converts to Compound III when combined with oxygen (Yamazaki and Yokota, 1965). The sudden decay of Compound III in the presence of low oxygen concentrations is the most interesting step in the periodic reaction. The mechanism of the reaction is not clear but it is most likely that the reaction is due to the reduction of Compound III by intermediates, such as the NAD radical and ferrous peroxidase (See Fig. 1). Compound III, thus, is thought to be a regulator of NADH oxidation by peroxidase.

It might be worth while to emphasize here that reduced pyridine nucleotide is the only substrate which causes the periodicity of the reaction among the well known peroxidase-oxidase substrates, such as dihydroxyfumarate, triose reductone and indoleacetate. It is obvious that a number of experiments remain to be done with the reduced pyridine nucleotide-peroxidase-oxygen system, particularly the demonstration of sustained periodicity of the reaction. This may be achieved when the reaction is carried out in the more complete open system which is now under investigation.

ACKNOWLEDGEMENTS

The authors wish to thank Professor L. H. Piette, University of Hawaii, for his helpful comments. The expert technical assistance of Mr. S. Miura and Mr. T. Arai for making a platinum electrode is greatly acknowledged. This investigation has been supported in part by Research Grant AM-06518 from the United State Public Health Service.

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