ISOLATION OF A NEW ELECTRON TRANSPORT COMPONENT WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-LIKE ACTIVITY

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The enzyme system in Mycobacterium phlei that is responsible for the endogenous reduction of 2,6-dichlorophenol indophenol (2,6-DPIP) (1) has been fractionated into two components (2). One of these is a glucose 6-phosphate dehydrogenase and the second a reduced coenzyme-2,6-DPIP reductase. The crude cell-free juice (3) contains the necessary coenzyme and substrate concentrations of glucose 6-P. The only added requirement for activation of the system is the addition of 2,6-DPIP as the final electron acceptor.

The nature of the components of the enzyme system suggested the addition of NADP. The level of enzyme activity obtained with added NADP was less than that observed following the addition of a boiled juice extract prepared from the crude cell-free juice. The extract was found to contain essentially no effective concentration of NADP as measured by a glucose 6-P dehydrogenase procedure. In addition no NAD was found using lactic dehydrogenase as the assay enzyme. These observations suggested that the natural coenzyme required by the enzyme system is not NADP nor NAD. In this communication a procedure for the isolation of a new electron transport component with NADP-like activity is described.

MATERIAL AND METHODS

The cell-free juice of Mycobacterium phlei was prepared as previously described (3) except the cell debris was removed by 30 minutes centrifugation at 32,700 X g in a Servall SS-1 centrifuge.

The fractions described below were assayed with a dialyzed, partly purified enzyme preparation. The purification of the enzyme system was carried to the point of lactic oxidative decarboxylase removal by isoelectric precipitation at pH 5.1 (1,4). The enzyme system was precipitated from the above supernatant solution by the addition of solid ammonium sulfate to a level of 0.6 saturation. The precipitate was removed and dissolved with the aid of 0.1M potassium phosphate buffer, pH 7.0. The resulting solution was dialyzed against deionized water for 24 hours.

The reduction of the 2,6-DPIP was measured at 600mμ in a Beckman DU spectrophotometer. Fluorescence was determined with a Type B Raymaster Lamp (3660A).

FRACTIONATION

Step 1 - One liter of the cell-free juice was heated for 10 minutes on a steam bath. Step 2 - The heated solution was rapidly cooled and one volume of acetone added. Super-Cel was added to aid in the filtration with vacuum. Step 3 - The filtrate was added to 3 volumes of absolute ethanol and the pH adjusted to 3.5 with 1N HCl. The resulting solution was stored at -20^{0} for 18 hours. Step 4 - The precipitate was collected and washed with cold acetone. The essentially dry precipitate was dissolved in water with the aid of 1N NaOH. Step 5 - The solution was adjusted to pH 3.5 with 1N HCl and the precipitate formed removed by centrifugation. The supernatant solution was added to five volumes of acetone and held at -20^{0} for 18 hours. Step 6 - The yellow waxy precipitate was collected and washed with acetone. The precipitate was dissolved with the aid of 1N NaOH. The resulting solution was frozen for storage.

RESULTS AND DISCUSSION

The level of the coenzyme during the course of the fractionation was measured with a dialyzed, partly purified enzyme system

consisting of glucose 6-P dehydrogenase and a reduced coenzyme-2,6-DPIP reductase. The preparation following Step 6 in the fractionation scheme contained both coenzyme and glucose 6-P.

Paper strip chromatography of this solution in a system designed to separate dinucleotides and consisting of ammonium sulfate added to 0.1M potassium phosphate buffer, pH 6.8 saturated with n-propanol revealed the presence of four substances that fluoresced blue in light of 3660A. Two yellow pigmented materials were located near the origin line. A small amount of two ultraviolet (2537A) absorbing substances, subsequently identified as NADP and thiamine pyrophosphate, were found on the strips.

The results of the enzymatic assay of the coenzyme solution are presented in Table 1.

TABLE 1

ASSAY OF COENZYME SOLUTION WITH PARTLY PURIFIED ENZYME SYSTEM

Minutes	No Additions	With G-6-P	With G-6-P And NADP	With G-6-P And NAD	With G-6-P And Coenzyme Solution
	Values expressed as O. D. at 600 mμ				
0.5	0.820	0.745	0.620	0.730	0.565
1.0	0.814	0.709	0.480	0.673	0.365
1.5	0.810	0.678	0.367	0.612	0.145
2.0	0.804	0.645	0.270	0.552	-
2.5	0.802	0.620	0.195	0.490	-
3.0	0.800	0.592	0.137	0.430	-

The cuvettes contained 4.5 mg of enzyme protein; 0.2 μmoles 2,6-DPIP; 2.0 ml 0.1M potassium phosphate buffer, pH 7.0; added where indicated: 0.10 μmoles NADP; 0.24 μmoles NAD; 0.1 ml coenzyme solution; 10 μmoles G-6-P; Q.S. 3 ml with deionized water.

It will be seen that the coenzyme solution substituted for NADP and increased the rate of reduction of the 2,6-DPIP considerably. The combination of the added glucose-6-P with that already contained in the coenzyme solution was not responsible for the increased reduction rate, since the enzyme system was completely saturated with substrate. The rate increase can therefore be at-

tributed to the suitability and concentration of the coenzyme. The level of NAD used was approximately 2.5 X that of the NADP. The data suggest that NADP is a more acceptable coenzyme than NAD.

Attempts to separate the components of the coenzyme solution (Step 6) with Dowex 1-chloride column chromatography and continuous-flow paper electrophoresis were not completely successful. It was determined that the mixture could be resolved on DEAE-cellulose activated with 0.01M sodium phosphate buffer, pH 7.0. The glucose-6-P passed directly through the column. The various components described above were eluted and examined in the assay system. The blue fluorescent components and a yellow pigmented material were inactive.

After large volumes of fluid had been passed through the column, a greenish-yellow fluorescent component was found at the top of the column. In white light this component was yellow. The colored DEAE-cellulose layer was removed and separated from the buffer by centrifugation. The resulting material was washed with deionized water and eluted by adjusting the pH to 11.5 with 1N NaOH. The eluate was filtered and the pH adjusted to 7 with 1N HCl.

The solution was assayed (Table 2). It will be seen that several additions of 2,6-DPIP can be made to the same reaction mixture with essentially the same result. The coenzyme eluted from DEAE-cellulose appears to effectively substitute for NADP in the enzyme system.

Paper strip chromatography procedures for dinucleotides and flavins exhibited a single spot with the material eluted from DEAE-cellulose, which did not correspond to any of the dinucleotides or flavins examined.

The coenzyme exhibited peaks at 260 and 420 mμ in 0.1 M potassium phosphate buffer, pH 7.0. On addition of acid (pH 2.5) the

yellow coenzyme solution became colorless. The 420 mμ peak disappeared and a new peak at 380 mμ was formed. The color returned and the 420 mμ peak was restored on readjusting the pH to 7.

TABLE 2

ASSAY OF THE DEAE-CELLULOSE FRACTIONATED COENZYME

WITH PARTLY PURIFIED ENZYME SYSTEM

Minutes	No Additions	With G-6-P	With G-6-P And NADP	With G-6-P and Coenzyme Dye Addition* (1)	(2)	(3)
	Values expressed as O.D. at 600 mμ					
0.5	0.780	0.730	0.639	0.620	0.601	0.620
1.0	0.775	0.700	0.503	0.478	0.459	0.487
1.5	0.770	0.670	0.390	0.335	0.325	0.364
2.0	0.770	0.637	0.292	0.210	0.205	0.260
2.5	0.765	0.609	0.213	0.092	0.104	0.165
3.0	0.765	0.508	0.150	0.026	0,046	0.093

* After the 2,6-DPIP was colorless (reduced), a second and then a third addition of dye was made to the same reaction mixture. The cuvettes contained the same materials as indicated in Table 1 except the coenzyme was added in a volume of 0.5 ml. The 260 mμ reading for this amount of coenzyme was 1.28 and that for the NADP added 0.62. 2,6-DPIP mixed with coenzyme did not change the O.D. at 600 mμ.

The coenzyme does not form a cyanide addition product characteristic of dinucleotides (5). Further examination of this point and other reactions must be delayed until additional supplies of the coenzyme are available.

REFERENCES

1. Sutton, W. B., Fed. Proc., 19,31 (1960).
2. Sutton, W. B., J. Bact., in submission.
3. Sutton, W. B., J. Biol. Chem., 210,309 (1954).
4. Sutton, W. B., J. Biol. Chem., 226,395 (1957).
5. Colowick, S. P., Kaplan, N. O., and Ciotti, M. M., J. Biol. Chem., 191,447 (1951).