PROPERTIES OF A NEW TPN-LIKE ELECTRON TRANSPORT COMPONENT FROM MYCOBACTERIUM PHLEI

W. B. SUTTON*

The Lilly Research Laboratories, Indianapolis, Indiana

Received March 12, 1964

Additional properties are herein noted for the new TPN-like electron transport component whose isolation was reported earlier in this journal (1).

FRACTIONATION:

The original 6 steps have been retained unmodified (1) and steps 7 to 11 added. Step 7 - The solution from Step 6 is melted and applie to a DEAE-cellulose column activated with 0.01 M sodium phosphate buffer, pH 7 and the fluorescent components previously described (1) washed through the column with 0.01 M phosphate buffer leaving the coenzyme as a fluorescent band at the top. Step 8 - The coenzyme was eluted with decreasing pH and increasing salt concentration gradient obtained by using 0.5 M NaCl in 0.1 M NaH_2PO_4. Step 9 - The eluate, neutralized to pH 7 with 0.1 N NaOH, is applied to a second DEAE-cellulose column activated with formic acid. The coenzyme is eluted with a gradient of 0.5 M ammonium formate in 0.75 M formic acid and neutralized to pH 7 with dilute NH_4OH. Step 10 - The eluate is lyophilized to reduce the volume, redissolved in 1/10th of the original volume and passed through a column of Amberlite IR-45 in the OH cycle to remove formate ion. Step 11 - The dissolved ammonia is removed from the effluent by holding under vacuum at 50°, and the resulting neutral solution is adsorbed on Barnebey-Cheney Carbon #1654

* Deceased March 25, 1964

(2). The carbon is washed repeatedly with deionized water, and then eluted with pyridine and water (1:1) made to pH 8.5 with dilute NH_4OH. The elution should be continued until no additional greenish-yellow coenzyme can be removed from the carbon. The pyridine is extracted with ethyl ether, and the resulting solution taken to dryness under vacuum at 50°. This dried preparation is extracted 3 times with absolute ethanol, the residual ethanol removed by vacuum, and the coenzyme dissolved in a small volume of water and the pH adjusted to 7 with 0.1 N NaOH. The resulting solution is frozen for storage. The greenish-yellow coenzyme may be precipitated from the aqueous solution as fine needle-like crystals by the addition of 5 volumes of acetone.

RESULTS AND DISCUSSION:

The purified greenish-yellow coenzyme exhibits several interesting spectral changes, as recorded by the Beckman Spectrophotometer, Model DB, over the pH range from 2.5 to 12.5 (Fig. 1 and 2). Of particular interest is the symmetrical shape of the single peak at 260 mμ (pH 2.5-3.0) and the minimum adsorption at 240 mμ (Fig. 1). In contrast at pH 7 and 12-12.5, the 260 mμ peak is replaced by a series of smaller peaks; and a shoulder is formed at 290 mμ. A major peak is formed at 240 mμ at pH 12-12.5, but not at pH 7.

The general nature of the peaks, their shift and form in acid and alkaline systems, resembles to some extent those obtained with pyridine or alkyl substituted pyridines. This point is under examination.

The electron transport property of the coenzyme resides in the colored chromophore (Fig. 3), which is believed to be linked to a pyridine-like structure by a carbon bridge. The arrangement being similar to FAD, where the adenosine moiety is associated with the isoalloxazine structure, but the latter is responsible for the electron transport function (3).

In the visible spectrum (Fig. 2) the greenish-yellow chromophore produces a peak at 420 mμ in alkaline and neutral solutions, which

shifts to 390 mμ at pH 2.5-3.0. Accompanying the shift in this peak is a loss of the greenish-yellow color with a transition point between greenish-yellow and colorless forms in the pH range of 4 to 5.

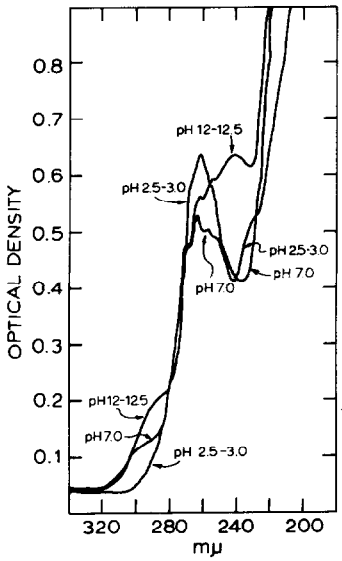

Fig. 1. Ultraviolet spectrum of the greenish-yellow coenzyme at several pH levels.

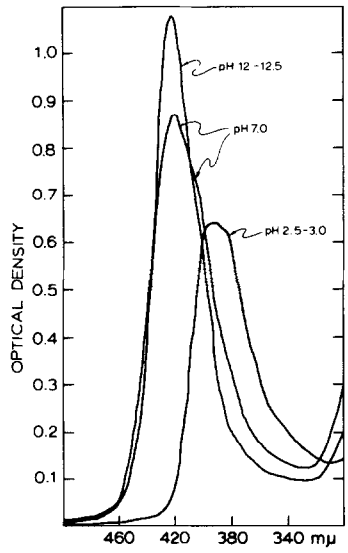

Fig. 2. Visible spectrum of the greenish-yellow coenzyme at several pH levels.

The greenish-yellow coenzyme can be reduced by substrate, i.e. glucose-6-phosphate in the absence of a final electron acceptor, i.e. 2,6-DPIP (Fig. 3). The reversible oxidation-reduction capacity of the coenzyme was reported (1) (Table 2, page 44).

The fluorescence spectrum of the greenish-yellow coenzyme was examined in the Aminco-Bowman Spectrophotofluorometer at pH 2.5, 7.0 and 11.5. At pH 2.5 the activating wave length is 400 mμ and the fluorescence peak at 445 ± 5 mμ. At pH 7 and 11.5 the activating wave length is 435 and the fluorescence peak at 485 ± 5 mμ. An examination of the publications of Udenfriend and his associates (4) failed to reveal any compounds of biological interest with a pattern similar to the greenish-yellow coenzyme.

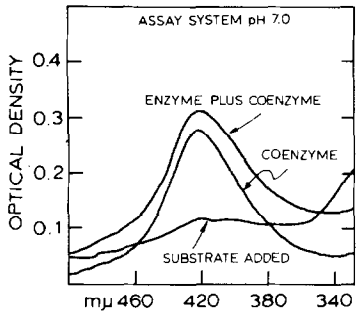

Fig. 3. Reduction of 420 mμ peak of coenzyme with added substrate in the absence of a final electron acceptor.

Bulen and Lecomte (5) reported the isolation of a yellow-green fluorescent peptide from spent iron-free medium following the growth of azotobacter. Dr. Bulen has kindly supplied both his yellow-green peptide and the crystalline chromophore obtained after the removal of the cyclic peptide by acid hydrolysis. These substances both exhibit a peak at 420 mμ in neutral and alkaline solutions; at pH 2.5, the peak shifts to 380 mμ, and in addition a peak at 240 mμ moves to 220 mμ (Fig. 2).

An unsuccessful attempt was made to substitute the yellow-green fluorescent peptide and the crystalline chromophore for the greenish-yellow coenzyme in the biological assay system (1). Recent mass spectrometer studies by Dr. Klaus Biemann have demonstrated that the crystalline chromophore of Bulen and the greenish-yellow coenzyme are not the same.

A standard procedure for determining the form of the coenzyme present in a flavin enzyme is to remove the coenzyme, and reactivate the resulting apoenzyme with FAD or FMN (6). Diaphorase (Clostridium kluyveri) was obtained from Worthington Biochemical Corp. An apoenzyme was prepared. The activity was determined with a DPNH-2,6-DPIP assay (5). The apoenzyme was activated by the addition of FAD and, to some extent, by FMN. The greenish-yellow coenzyme failed to reactivate the apoenzyme of diaphorase.

In summary the coenzyme is not structurally related to the known coenzymes, nor does it contain moieties associated with known coenzyme function.

Preliminary work on the mechanism of action of the coenzyme has established a view somewhat different than that originally held. A

SYSTEM A

$$\text{G-6-P} + \underset{\text{(oxid)}}{\text{CoEz}} \xrightarrow{\text{G-6-P DH}} \text{6-PG} + \underset{\text{(reduced)}}{\text{CoEz}}$$

$$\underset{\text{(reduced)}}{\text{CoEz}} + \underset{\text{(oxid)}}{\text{2,6-DPIP}} \xrightarrow{\text{Reductase}} \underset{\text{(oxid)}}{\text{CoEz}} + \underset{\text{(reduced)}}{\text{2,6-DPIP}}$$

SYSTEM B

$$\text{G-6-P} + \underset{\text{(oxid)}}{\text{XCoEz*}} \longrightarrow \text{6-PG} + \underset{\text{(reduced)}}{\text{XCoEz}}$$

$$\underset{\text{(reduced)}}{\text{XCoEz}} + \text{2,6-DPIP} \longrightarrow \underset{\text{(oxid)}}{\text{XCoEz}} + \underset{\text{(reduced)}}{\text{2,6-DPIP}}$$

* XcoEz = apoenzyme plus coenzyme

dialyzed, partly purified enzyme mixture (1) containing both a G-6-P dehydrogenase and a reduced coenzyme-2,6-DPIP reductase was used in making the original observation that the greenish-yellow coenzyme could be substituted for TPN (SYSTEM A). When this system was reconstructed with DEAE-cellulose purified G-6-P dehydrogenase and the reductase (7), the addition of G-6-P and TPN activated the system, however the greenish-yellow coenzyme failed to substitute for the TPN. These conflicting results led to the conclusion that a single enzyme, functioning in a manner similar to a flavin enzyme, could be responsible for the activity obtained in SYSTEM B.

In support of this view is the fact that isocitric dehydrogenase does not function with the greenish-yellow coenzyme, but does with TPN. A similar statement can be made for 6-PG dehydrogenase activity. If the greenish-yellow coenzyme were truly a universal substitute for TPN, both of these systems would have been activated by the coenzyme.

The greenish-yellow coenzyme requires G-6-P rather than glucose as substrate. Further examination of the one enzyme concept and other reactions must be delayed until enzyme purification is achieved.

ACKNOWLEDGMENT

The author would like to thank Dr. Klaus Biemann for the mass spectra observations.

REFERENCES

1. Sutton, W. B., Biochem. Biophys. Res. Comm., 10, 40 (1963).
2. Bartlett, G. R., J. Biol. Chem., 234, 459 (1959).
3. Dixon, M., and Webb, E. C., In Enzymes, Academic Press Inc., N.Y., p. 436 (1958).
4. Duggan, D. E., Bowman, R. L., Brodie, B. B., and Udenfriend, S., Arch. Biochem. Biophys., 68, 1 (1957).
5. Bulen, W. A., and Lecomte, J. R., Biochem. Biophys. Res. Comm., 9, 523 (1962).
6. Koike, M., Shah, P. C., and Reed, L. J., J. Biol. Chem., 235, 1939 (1960).
7. Sutton, W. B., J. Bact., 85, 476 (1963).