PHYSICAL PROPERTIES OF PHOTOSYNTHETIC PYRIDINE NUCLEOTIDE REDUCTASE[1]

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The reduction of triphosphopyridine nucleotide (TPN) by illuminated chloroplasts is most probably the consequence of a multi-component (or multi-reaction) process. To date, very little is known concerning the number of intermediate steps required in the formation of reduced TPN at the expense of light energy.

Studies in this laboratory have established that the photochemical reduction of TPN requires both chloroplasts and a soluble factor isolated originally from chloroplasts (San Pietro and Lang, 1958). This finding represented the first demonstration that a soluble factor can be added back to chloroplasts to reconstitute their over-all electron transport reaction. The soluble enzyme, photosynthetic pyridine nucleotide reductase (PPNR), has been purified extensively and it has been shown (Keister et al., 1961) that, under the proper conditions, the reduction of TPN proceeds at a rate (about 325 μmoles per hour per mg. of chlorophyll) commensurate with that of photosynthesis (Rabinowitch, 1956).

The elucidation of the mechanism of action of PPNR is basic to an understanding of the mechanism of reduction of TPN. Toward this end, it was desirable to gain some information on the physical properties of the enzyme. In this report are presented the results of this study.

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MATERIALS AND METHODS

The enzyme was purified as previously described (San Pietro, 1961). Enzymatic activity was determined spectrophotometrically by measuring the initial rate of formation of reduced TPN in the Hill reaction (San Pietro, 1961). Protein concentration was determined by the Folin Phenol method (Lowry et al., 1951).

Free electrophoresis was carried out in a Perkin-Elmer Tiselius apparatus, Model 38, with a 2 ml. cell. Zone electrophoresis on a Munktell's cellulose powder column was performed at 4° essentially as described by Porath (1956).

Sedimentation was observed in a Spinco Model E analytical ultracentrifuge, equipped with Schlieren optics and automatic rotor temperature control, at a rotor speed of 59,780 rpm. Sedimentation coefficients were corrected to a standard state in water at 20°.

Diffusion measurements were made with a synthetic boundary cell (Pickels et al., 1952). The diffusion coefficient of the protein was estimated by the use of the first moment method. The partial specific volume of the protein was assumed to be 0.71 and this value was used in all calculations.

Molecular weight measurements were performed by the method of Ehrenberg (1957) with a 12 mm synthetic boundary cell with a rubber valve and a rotor speed of 23,150 rpm. Viscosity measurements were made with an Ostwald-Fenske viscosimeter, of 2 ml volume, in a constant temperature bath maintained at 20 ± 0.03°.

RESULTS AND DISCUSSION

Fig. 1 illustrates the nature of the pattern obtained during ultracentrifugation and demonstrates that the material sediments as a single weight class. A single symmetrical peak was apparent at the protein concentrations tested; namely, 0.28, 0.59, 0.74 and 0.92 per cent. The extrapo-

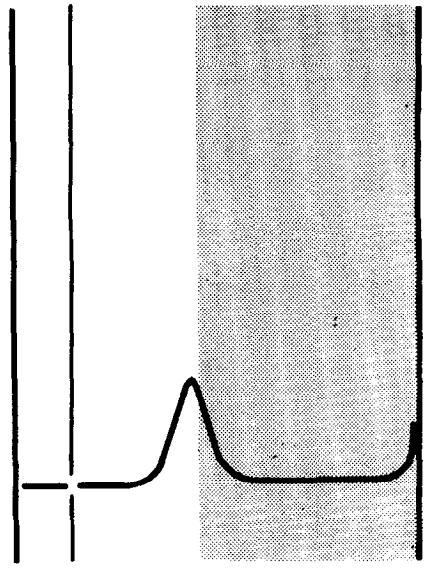

Fig. 1 Schlieren pattern in a synthetic boundary cell. Protein concentration was 7.4 mg./ml. Phosphate buffer, pH 7, at an ionic strength of 0.1 was used.

lated value for the sedimentation coefficient of this single peak was 1.36 Svedbergs. It may be of interest that the observed sedimentation coefficient increased slightly in a linear fashion with increasing protein concentration and may reflect some association of the protein at the higher concentrations. The relationship between the observed sedimentation coefficient, S, and protein concentration, c, in per cent is given by the equation $S = 1.36\ (1 + 0.25\ c)$.

It should be noted that the purified PPNR is reddish-brown in color (San Pietro, 1961). It is clear from Fig. 1 that the color remains associated with the protein during ultracentrifugation. Under acidic conditions (pH 2) the enzyme is irreversibly bleached and there is a concomitant loss of activity. Sedimentation analysis of this material indicated a high degree of polymerization with a mean value for the sedimentation coefficient of about 6 Svedbergs.

The diffusion coefficient was estimated to be 6.60×10^{-7} cm^2 sec^{-1} at zero protein concentration. A plot of the value of the diffusion coefficient versus protein concentration yielded a straight line with zero slope. Using the experimentally determined values for the sedimentation and diffusion coefficients and the assumed partial specific volume, the molecular weight of the enzyme was calculated by the Svedberg equation to be 17,000. This value is given strong support by the results of the equilibrium studies. The molecular weight measured at five successive intervals during equilibrium centrifugation remained constant at $17,000 \pm 2,000$. The constancy of this value is additional evidence for the homogeneity of the preparation.

Viscosity measurements were carried out in 0.005 M Tris, pH 8. The reduced viscosity was estimated to be 0.150 dl/g at zero protein concentration. Assuming the particle to be an anhydrous prolate ellipsoid, the axial ratio calculated from the Einstein viscosity increment ($\nu = 21$) was estimated to be 14. This is in reasonable agreement with the axial ratio calculated from sedimentation and diffusion data using the Perrin equation ($b/a = 18$). These values seem high for a protein of such low molecular weight.

As stated above, the enzyme is colored and the ratio of optical density at 280 mμ to that at 260 mμ is about 1.2 (San Pietro, 1961). This low ratio is most probably due to the chromophore but may be due to a contamination with nucleic acid to the extent of about 1.5 per cent. These alternative possibilities are under investigation.

In Fig. 2 is shown the elution diagram obtained in zone electrophoresis. One single and symmetrical peak is present at the pH and concentration used. The results in free boundary electrophoresis at three different pH's (6, 7 and 8) also show a single peak with no evidence of other components. These experiments were carried out in phosphate buffer, of ionic strength of 0.1, at 4°. At pH 7, the mobility was calculated to be 7.57×10^{-5} cm^2 sec^{-1} $volt^{-1}$. In acetate buffer, pH 5, it is possible

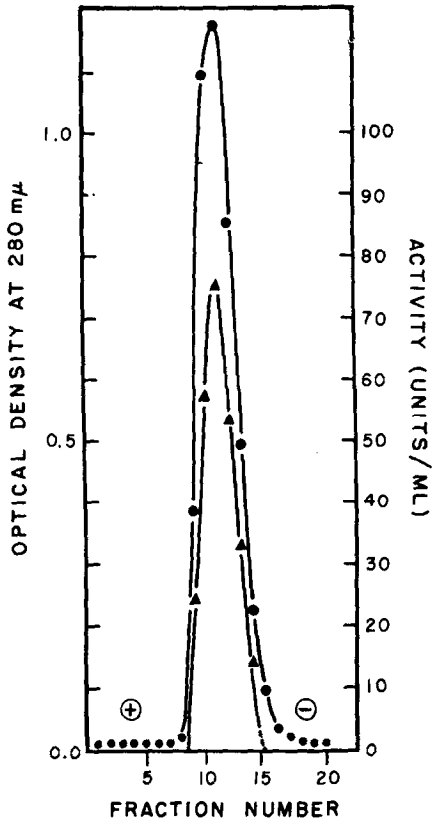

Fig. 2 Cellulose column electrophoresis of PPNR. The size of the column was 45 x 2.5 cm; the current was 25 mA; and the time was 18 hours. Phosphate buffer, pH 8, at an ionic strength of 0.03 was used. (•) Optical Density; (▲) Activity.

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to observe the presence of a small second component, the percentage of which appears to increase in proportion to the loss in enzymatic activity.

Davenport (1960) has reported that the "methemoglobin-reducing factor," shown previously to catalyze the reduction of methemoglobin and metmyoglobin by illuminated chloroplasts, is also active in catalyzing the reduction of TPN in this system. The molecular weight of the protein, calculated from sedimentation and diffusion measurements, has been reported to be 19,000 (Davenport and Hill, 1960). In view of this, and other similarities between PPNR and the "methemoglobin-reducing factor," it may be that these two activities are associated with the same protein.

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REFERENCES

Davenport, H. E., Biochem. J. 77, 471 (1960).

Davenport, H. E., and Hill, R., Biochem. J. 74, 493 (1960).

Ehrenberg, A., Acta Chem. Scand. 11, 1257 (1957).

Keister, D. L., San Pietro, A., and Stolzenbach, F. E., Arch. Biochem. Biophys. 94, 187 (1961).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

Pickels, E. G., Harrington, W. F., and Schachman, H. K., Proc. Natl. Acad. Sci. U.S., 38, 943 (1952).

Porath, J., Biochim. et Biophys. Acta 22, 151 (1956).

Rabinowitch, E. I., "Photosynthesis and Related Processes," Vol. 2, Pt. 2, p. 1594, Interscience Publishers, New York (1956).

San Pietro, A., in "Light and Life" (McElroy, W. D. and Glass, B., eds.), p. 631, Johns Hopkins Press, Baltimore, 1961.

San Pietro, A., and Lang, H. M., J. Biol. Chem. 231, 211 (1956).