STUDIES ON PHOTOSYNTHETIC PYRIDINE NUCLEOTIDE REDUCTASE [1]

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Previous reports from this laboratory (San Pietro, 1961; Appella and San Pietro, 1962) have described properties of a reddish-brown protein isolated from spinach, photosynthetic pyridine nucleotide reductase (PPNR), which catalyzes the photochemical reduction of TPN and certain haem-proteins in the presence of chloroplasts. Purified preparations of this enzyme have been reported to contain non-haem iron (Katoh and Takamiya, 1962; Lazzarini and San Pietro, 1962).

In collaboration with Dr. B. L. Vallee, Harvard Medical School, a spectrographic metal analysis has been made of fractions obtained during the purification of PPNR through the protamine step described by San Pietro and Lang (1958) and finally by chromatography on DEAE cellulose by the method of Hill and Bendall (1960). Throughout the purification, precautions were taken to minimize the introduction of extraneous metal ions. The results obtained (Fry et al., in preparation) show that iron is concentrated during purification of the enzyme. Iron was the only metal found in the purified enzyme in significant concentration.

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Based on a molecular weight of 17,000 (Appella and San Pietro, 1962) and protein concentration as determined by dry weight measurement on material precipitated by 10% TCA, the purified enzyme contained 2.2 gram-atoms of iron per mole of enzyme. At present, however, great significance cannot be ascribed to this value. Variations in iron content among different preparations have been noted. In addition, values of protein concentration as determined by different methods are not in agreement. Consequently it is impossible for us to determine an exact molar ratio for iron to protein on the basis of evidence at hand. Katoh and Takamiya (1962) have reported a value of 4 for this molar ratio but the method of determination was not indicated.

Although treatment of PPNR with the iron chelating agent o-phenanthroline (OP) at pH 5 at ca. 80° for 5 minutes results in the liberation of 97 - 100% of the iron present as the ferrous triphenanthrolate complex, $Fe(OP)_3^{++}$, preliminary experiments indicate that the iron in the enzyme is in the ferric form and that reduction to the ferrous state occurs during or after release from the enzyme.

In addition to iron, PPNR contains "labile sulfur" which is liberated as hydrogen sulfide upon acidification. The odor of hydrogen sulfide can also be detected following prolonged incubation of enzyme with OP at pH 8. Assays for the sulfide were performed by applying the method of Fogo and Popowsky (1949) directly to solutions of the protein. The molar ratio of iron to labile sulfur with several preparations varied from 0.93 to 1.6. Control experiments indicate that cysteine groups in the protein are not the source of the labile sulfur.

To determine the changes resulting from treatment with OP, the enzyme was incubated with the chelator and then, at varying time intervals, separated from the latter, as well as from the ferrous triphenanthrolate which formed,

by chromatography on Sephadex G-25. Analyses of protein fractions obtained in such an experiment are summarized in Table 1. The data indicate that under conditions of pH and temperature at which PPNR is relatively stable, treatment with OP results in loss of iron from the enzyme as well as loss of labile sulfur, absorption in the visible region of the spectrum and activity of the enzyme in TPN and cytochrome c reduction.

The correlation between the percentage of iron lost and the loss of absorption in the visible region of the spectrum is actually better than is apparent from the values reported in Table 1 since there is apparently some residual absorption in this region even in iron-free enzyme. The origin of this residual absorption is currently under investigation as is the relationship of the labile sulfur to the activity of the enzyme.

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TABLE 1

Properties of PPNR following incubation at 25° and pH 8 in the presence or absence of o-phenanthroline[a]

Sample -OP	Sample +OP	Reaction time in minutes	Percentage retention of[b] Iron[c]	Labile sulfur	Activity in TPN reduction[d]	Activity in cytochrome c reduction[e]	Absorption Maxima at: 329mμ	420mμ	465mμ
1		7	93	92	93	100	99	100	101
2		264	92	80	85	84	92	90	90
	3	7	89	84	90	101	94	94	95
	4	46	65	67	66	66	75	73	73
	5	92	51	51	55	49	61	58	57
	6	142	37	37	34	31	49	42	41
	7	248	17	15	12	13	32	22	21
	8	ca. 1400	4	--	--	--	17	5	4

a. The reaction mixture consisted of 20.1 mg of PPNR (by Folin assay), 235 μ moles of Tris buffer, pH 8, and 15 μ moles of OP in a final volume of 4.65 ml. The PPNR used contained 2.3 μ moles of iron and 2.2 μ moles of labile sulfur. Aliquots of 0.6 ml were removed for chromatography on columns of Sephadex G-25 which had been equilibrated with 0.05 M Tris buffer, pH 8. The control mixture was of similar composition but contained no OP.

b. Percentages were calculated on the basis of equal amounts of protein by Folin estimation.

c. Measured as $Fe(OP)_3^{++}$ after heating sample at ca. 80° for 5 minutes at pH 5 in the presence of excess OP.

d. Measured according to San Pietro (1958).

e. The non-photochemical reduction of cytochrome c as measured by the method of Lazzarini and San Pietro (1962).