FREEDOM OF HYDROXYPROLINE-2-EPIMERASE FROM PYRIDOXAL PHOSPHATE*

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It is generally postulated that amino acid racemases involve a pyridoxal phosphate mechanism, although this thesis is supported directly by only a few studies with significantly purified enzymes (Wood, 1955; Behrman, 1962). Indirect support comes from the nonenzymatic racemization of certain amino acids catalyzed by pyridoxal (Olivard et al., 1952). Observations with certain partly purified racemases (Stadtman and Elliott, 1957; Glaser, 1960; Tanaka et al., 1961), however, have failed to implicate pyridoxal phosphate in the reaction mechanism, although no definitive evidence excluding this enzyme has appeared in studies of any specific amino acid racemase. In part, uncertainty rests on failure to demonstrate high absolute purification in amino acid racemases previously described, although several-hundred fold relative purification has been obtained in some cases (Glaser, 1960; Tanaka et al., 1961).

An inducible enzyme from Pseudomonas striata that equilibrates epimers of hydroxyproline by inversion of configuration at carbon 2 was briefly described earlier (Adams, 1959). This

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enzyme, hydroxyproline-2-epimerase, has since been purified to homogeneity or near-homogeneity by the criteria of monodisperse sedimentation in the analytical ultracentrifuge and electrophoretic migration in starch gel as a single band (associated with enzyme activity) over a 2.5 pH-unit range. Although the degree of relative purification (200-fold) needed to achieve homogeneity is low, it is consistent with other experience in the purification of induced or derepressed enzymes (Cohn, 1957; Sheperdson and Pardee, 1960).

Accepting the cited criteria of homogeneity, direct evidence against pyridoxal phosphate as an obligatory component of the active enzyme has been obtained by determination of enzyme phosphorus as P^{32}. In separate experiments, two 12-liter cultures were grown on 10 and 20 mc respectively of inorganic P^{32} phosphate (composition of medium and growth conditions have been described (Adams, 1959)). Purified enzyme obtained from P^{32}-labeled cells represented approximately 1 mg per liter, and migrated as a single band on starch gel. Although the enzyme as obtained by the routine purification procedure contains variable quantities of phosphorus both by chemical and isotope assay, several procedures remove all but traces of phosphorus with little or no loss of enzymatic activity. These include treatment with Dowex-1 acetate, prolonged dialysis, or centrifugation through sucrose gradients. The removal of P^{32} by treatment with Dowex-1 is outlined in Table 1.

Other observations are also inconsistent with pyridoxal as a cofactor. Several carbonyl reagents (hydroxylamine,

TABLE I

Phosphorus Content of Purified Hydroxyproline-2-epimerase.

Enzyme purified from cells grown in P^{32}-containing media was stirred for 5 minutes at 0° with 6 mg/ml of dry Dowex-1-X8 (acetate). The resin was removed by centrifugation. Enzyme activity was measured by a polarimetric method (1 unit catalyzes the conversion of 1 μmole of allohydroxy-D-proline to hydroxy-L-proline per minute under specified assay conditions); radioactivity was measured in an end-window gas-flow counter; protein was measured turbidimetrically. All values are based on 1 ml of enzyme.

	Before Dowex-1	After Dowex-1
Enzyme Units	185	139
c.p.m.	2310	90
Protein (mg)*	2.8	2.3
P (μatoms)**	0.089	0.0035
Protein (μmoles)*	0.16	0.13
μatoms P/μmole protein	0.56	0.027

* The molecular weight of hydroxyproline-2-epimerase has been estimated as approximately 18,000 by the Archibald method (Schachman, 1957), assuming a partial specific volume of 0.75. Molar concentrations of enzyme solutions are calculated from this value and from the protein concentration obtained by a turbidimetric method (Stadtman et al., 1951) using crystalline beef serum albumin as standard.

**Specific activity of medium phosphorus at this time was 26,000 c.p.m./μatom.

hydrazine, semicarbazide, isonicotinyl hydrazide, cyanide) failed to produce inhibition of enzyme activity at concentrations lower than 0.1 to 0.01 M. The enzyme spectrum shows no indication of pyridoxal absorption at pH 3.3, pH 7.5 or pH 9.8 (Fig. 1). Efforts to produce nonenzymatic racemization of

hydroxy-L-proline with pyridoxal or pyridoxal phosphate under conditions producing over 50% racemization of L-alanine (Olivard et al., 1952) failed to show detectable formation (less than 1%) of allohydroxy-D-proline.

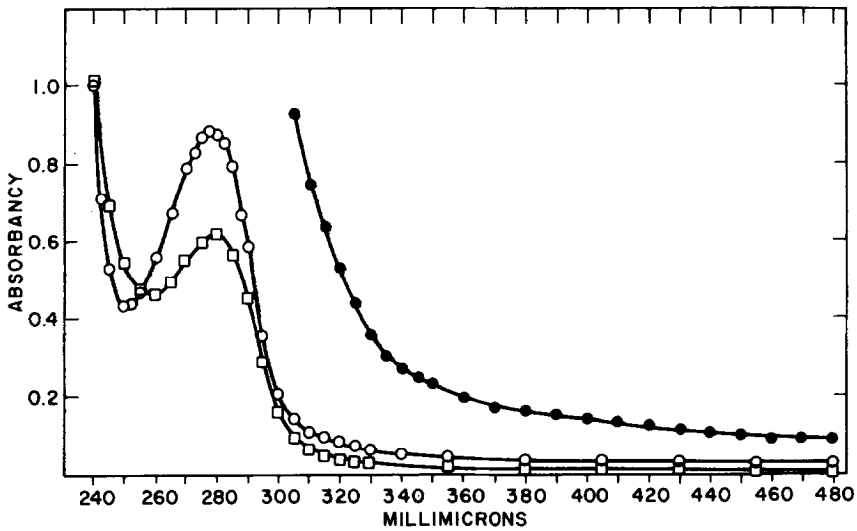

Fig. 1. Spectra of purified hydroxyproline-2-epimerase. Open circles: protein concentration 0.9 mg/ml in 0.01 M phosphate, pH 7.5. Open squares: protein concentration 0.9 mg/ml in 0.05 M glycine, pH 9.8. Solid circles: protein concentration 3.8 mg/ml in 0.001 M phosphate, pH 7.5. Enzyme samples adjusted to pH 3.3 with acetic acid gave essentially superimposable spectra with those at pH 7.5. Addition of substrate (0.05 M allohydroxy-D-proline) produced no change in the spectrum of the enzyme at 1.2 mg/ml and pH 7.5. All spectra were obtained with a Zeiss PMQ spectrophotometer in 1 cm cells at room temperature. Enzyme samples were read against cuvets containing the same buffer. All solutions contained 0.0005 or 0.001 M neutral EDTA.

A relevant feature of the enzyme-catalyzed reaction is the incorporation of 1 μatom of tritium from water per μmole of hydroxyproline epimerized. Although this would be demanded by a pyridoxal-Schiff base model, it is also consistent with other mechanisms, including a dehydrogenated intermediate or a direct displacement reaction.

The demonstration that hydroxyproline appears to be racemized at the α-carbon by an enzyme free of pyridoxal phosphate provides a positive example for Braunstein's generalization (1960) that pyridoxal enzymes are not known that catalyze reactions with substituted amino groups. Consistently, proline racemase, (Stadtman and Elliott, 1957), although not highly purified, was not inhibited by ultraviolet light or hydroxylamine, and efforts to demonstrate a pyridoxal phosphate requirement were unsuccessful.

That the participation of pyridoxal phosphate in racemizing enzymes is more likely to be a function of the substrate than the organism is suggested by preliminary observations with alanine racemase from cells of hydroxyproline-grown P. striata. In contrast to hydroxyproline-2-epimerase in the same preparations, alanine racemase is strongly inhibited by 10^{-3} M hydroxylamine and after partial purification is stimulated by pyridoxal phosphate.

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