Isolation and Characterization of Enolase from Rainbow Trout (*Salmo gairdnerii gairdnerii*)*

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ABSTRACT: Enolase has been purified and crystallized from the white skeletal muscle of rainbow trout. The crystalline enzyme contains three electrophoretically distinct enolases, and since the same ratio of these three enzyme forms was present in the original muscle extract, it is concluded that the three forms are not artifacts of isolation. Working with the mixture of the three forms, the trout muscle enolase has been characterized with respect to physical, chemical, and biochemical properties.

The crystalline enzyme was homogeneous in the ultracentrifuge and had a molecular weight of 91,000. In contrast with enolases from yeast and rabbit, it contains both sulfhydryl and disulfide groups. Its catalytic properties are very similar to other enolases both with respect to kinetic constants and inhibition by fluoride, and with respect to the absolute requirement for divalent metal ion.

The skeletal muscle of several species of Salmonidae has been reported to contain multiple forms of the common glycolytic enzyme enolase(2-phospho-D-glycerate-hydro-lyase (4.2.1.11)) (Tsuyuki and Wold, 1964). In an attempt to investigate this phenomenon in some detail in terms of both the structural and functional significance of the different forms of enolase, a pure preparation of enolase must first be obtained. This paper reports the purification and crystallization of enolase from the white skeletal muscle of rainbow trout (*Salmo gairdnerii gairdnerii*) and some preliminary data on the characterization of the enzyme. The three forms of the enzyme which can be detected in the original muscle extract (Tsuyuki and Wold, 1964) are present in the crystalline enzyme preparation in what appears to be unchanged proportions, and the biochemical, chemical, and physical properties reported in this paper are determined on the mixture of the three forms.

Experimental Procedure

Assays. Enolase activity was determined by the direct spectrophotometric method of Warburg and Christian (1942), according to published procedures (Holt and Wold, 1961) using a Zeiss or a recording Gilford spectrophotometer. Protein determinations were based on the 280-mμ absorbance and related to accurate dry weight of the pure enzyme.

The barium salt of the substrate (D-glyceric acid 2-phosphate, Sigma Chemical Co.) was converted to the water-soluble cyclohexylammonium salt (Winstead and Wold, 1966), imidazole was recrystallized twice from benzene after treatment with charcoal, and Tris was recrystallized from hot methanol–water. All other reagents were of the highest available purity. Glass-distilled water was used throughout.

Physical Methods. Sedimentation equilibrium and velocity runs were carried out according to standard procedures (Schachman, 1957; Richards and Schachman, 1959) using the Spinco Model E ultracentrifuge equipped with schlieren and interference optical systems. A partial specific volume of 0.728 (Winstead and Wold, 1965) was assumed for all calculations. Starch gel electrophoresis was done with the equipment and according to the procedures described by Tsuyuki (1963). The protein samples were dialyzed against the electrophoresis buffer for several hours before being applied to the gel. A horizontal section of the gel was stained with amido black (Tsuyuki, 1963). For the purpose of determining which of the stained protein bands corresponded to enolase, a uniform cylinder of gel was removed from appropriate locations in the unstained counterpart of the gel with a flat, 13-gauge hypodermic needle. The cylinder was mascerated with a polyethylene rod in a cuvet containing 3 ml of assay medium, and the starch particles were allowed to settle for about 30 sec. At this time it was possible to start the readings of the 240-mμ absorbance (enolpyruvic acid phosphate formation), and the absorbance change was followed for the next 2–4 min. The results from these determinations were surprisingly reproducible, and checked well with other more quantitative results (Tsuyuki and Wold, 1964).

Chemical Methods. Amino acid analyses were performed with the Spinco amino acid analyzer Model 120B (Moore *et al.*, 1958) after acid hydrolysis for 21 hr at 110° in sealed and evacuated (50 μ) tubes. Correction for destruction during hydrolysis was based

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on recovery values from standard amino acid mixtures hydrolyzed under identical conditions. The total content of cystine and cysteine was determined as cysteic acid after performic acid oxidation (Hirs, 1956). Tryptophan was determined by the method of Spies and Chambers (1948) in samples of enolase digested for 24 hr with 1% (w/w) of pronase at pH 7.0 and 40°. Free sulfhydryl content both before and after reduction with sodium borohydride (at pH 9) was determined by spectrophotometric titration with *p*-MB[1] in 8 M urea at pH 5.0 (0.1 M acetate buffer) (Boyer, 1954).

Preparation of Enzyme. The purification procedure is similar to the one developed for rabbit muscle enolase (Winstead and Wold, 1966). Frozen whole rainbow trout (from Roundhouse Trout Farm, Parchment, Mich.) was partially thawed and cleaned. Fillets freed from the thin layer of red muscle immediately under the skin were refrozen in packages of 500–700 g. These packages have been stored for several months without any apparent qualitative or quantitative change in the muscle enolase, and the enzymes isolated from this starting material were indistinguishable from the enzymes isolated from freshly killed fish.

STEP 1. EXTRACTION. The frozen fish was ground once in the meat grinder while still frozen and then blended with two parts (by weight) of cold EDTA solution (0.05% (w/v) of EDTA tetrasodium salt) for 5 min in the cold. The homogenate was immediately centrifuged at 10,000*g* for 1 hr in a refrigerated centrifuge at 0°. Small amounts of flocculent material was removed from the supernatant by filtration through glass wool.

STEP 2. ACETONE FRACTIONATION. Reagent grade acetone was precooled to −10° and the appropriate amount (540 ml/1000 ml of extract) was added rapidly to the extract with efficient stirring at 4°. After centrifugation at 10,000*g* for 1 hr at −5°, more acetone (313 ml/l. of original extract) was added to the decanted supernatant, and the solution was again centrifuged at 10,000*g* for 1 hr at −5°. The supernatant was removed as completely as possible from the precipitate by decantation, draining, and finally by wiping the centrifuge tubes with tissue, and the resulting precipitate was dissolved in cold imidazole–Mg buffer (0.05 M imidazole–0.001 M $MgSO_4$, pH 7.9); approximately 100 ml/1000 ml of original extract. Assuming the acetone–water volumes to be additive this acetone step collects the proteins precipitating between 35 and 46% acetone. Prolonged exposure to these acetone concentrations leads to loss of activity.

STEP 3. HEAT TREATMENT. To the solution of the acetone fraction was added the appropriate volume of a 50% (w/v) solution of $MgSO_4 \cdot 7H_2O$ to give a 0.5% (w/v) concentration of Mg salt in the protein solution. This solution was stirred overnight in a shallow dish in the 4° room to remove the last traces of acetone. The volume at this point was about 110 ml/1000 ml of original extract. The solution was rapidly brought to 55° by immersion in a 70° water bath with vigorous swirling, was held at 55° for 3 min, and was then quickly cooled in an ice bath to at least 10°. The voluminous precipitate was removed by centrifugation at 10,000*g* for 1 hr at 0° and the clear supernatant collected.

STEP 4. AMMONIUM SULFATE FRACTIONATION. It must be emphasized that whenever the enzyme was exposed to concentrated ammonium sulfate solutions, it was found essential to exclude oxygen. In the presence of air, activity was rapidly lost during the ammonium sulfate fractionation. Thus, in all the manipulations involving ammonium sulfate solutions, all solutions were carefully purged with nitrogen gas and kept in tightly stoppered vessels under a nitrogen atmosphere. Solid ammonium sulfate (Mallinckrodt, analytical reagent) was added to the heat supernatant at 4° to bring the concentration to 55% saturation (350 g/l. of supernatant). The solution was stirred for 15 min at 4° and then centrifuged at 10,000*g* for 1 hr. The supernatant was brought to 75% saturation by the further addition of solid ammonium sulfate (169 g/l. of heat supernatant). The solution was again stirred and centrifuged as above. (The values for per cent saturation are based on saturation at 25°.) An ammonium sulfate solution was prepared by dissolving 760 g of ammonium sulfate and about 100 mg of $MgSO_4 \cdot 7H_2O$ in 1 l. of water and after addition of concentrated ammonium hydroxide to pH 7.9, allowing the excess ammonium sulfate to crystallize out at 4° for at least 2 days. This 4°, saturated ammonium sulfate solution was used in the subsequent steps. The precipitate from the above salt fractionation was extracted with 25 ml/100 ml of heat supernatant of a solution of three parts of 4°, saturated ammonium sulfate and two parts of imidazole buffer (0.05 M imidazole–0.001 M $MgSO_4$, pH 7.9) by gentle stirring overnight, and the precipitate remaining after centrifugation at 10,000*g* for 1 hr was reextracted in the same manner. After two extractions, very little activity remained in the precipitate which was discarded.

STEP 5. CRYSTALLIZATION. To the two extracts was added sufficient 4°, saturated ammonium sulfate solution (usually about 1 ml) to cause slight turbidity, and crystal formation would generally start within a few minutes. Optimal yields of crystals were obtained after 1–3 days by centrifugation at 10,000*g* for 1 hr at 4°.

STEP 6. RECRYSTALLIZATION. The crystalline enzyme was dissolved in a minimum amount of imidazole buffer (0.05 M imidazole–0.001 M $MgSO_4$, pH 7.9) and after addition of 4°, saturated ammonium sulfate solution till slight turbidity appeared, the crystallization was allowed to proceed as before. From this point fractions with similar specific activity were combined. Maximum specific activity and purity (as determined by starch gel electrophoresis) were obtained after two or three recrystallizations. Most of the experiments were carried out using this recrystallized enzyme, but for the determination of the hydrodynamic properties, a further purification step was necessary to remove a small

[1] Abbreviation used: *p*-MB, *p*-mercuribenzoate.

amount (5%) of high molecular weight material. The crystals can be stored in 70% saturated ammonium sulfate under nitrogen. In the presence of air the activity is lost over a period of a few weeks.

STEP 7. CHROMATOGRAPHY ON SEPHADEX G-100. A 120 × 2.1 cm column of Sephadex G-100 (with fines removed) was equilibrated at 4° with 0.025 M Tris phosphate buffer, pH 8.6, containing 10^{-3} M magnesium sulfate. A concentrated solution of the protein was dialyzed against two changes of the same buffer at 4°. About 200 mg of protein in a final volume of 1–1.5 ml was fractionated on the column at a flow rate of 30 ml/hr, collecting 7.5-ml fractions.

Results

The results of the enzyme purification are summarized in Table I, and the electrophoretic pattern of the crystalline enzyme is given in Figure 1. The three protein bands all have enzymatic activity and their electrophoretic properties appear to be identical with those present in the original muscle extract (Figure 1). Attempts to isolate the individual forms of the fish muscle enolase by preparative starch gel electrophoresis always gave products with greatly diminished specific activity and contaminated with starch, even after treatment with amylase. Except for some individual amino acid analyses, the characterization of the rainbow trout enolase was consequently carried out with the mixture of the three enzyme forms. The difference in mobility of the enolases in the two buffer systems in Figure 1 is very dramatic, and appears to be characteristic of several fish enolases.

The molecular weight of the enzyme was determined by equilibrium sedimentation in the Spinco Model E ultracentrifuge using interference optics (Richards and Schachman, 1959). The centrifuge speed was 5325 rpm and two different protein concentrations in 0.025 M Tris phosphate buffer, pH 8.6, were used. The molecular weights from three runs were found to be 90,000 and 91,400 (at 5.3 mg/ml) and 90,8000 (at 2.6 mg/ml). When the natural logarithm of the interference fringe number was plotted against the square of the radial distance to that fringe, a straight line resulted, demonstrating the weight homogeneity of the enzyme (Figure 2). Homogeneity is also indicated by the insert in Figure 2 showing the schlieren pattern obtained with a 11-mg/ml sample in the same buffer in a sedimentation velocity run. The sedimentation coefficient was found to be $s_{20,w} = 5.5$ S. All of these experiments were performed on enzyme purified by gel filtration on Sephadex G-100. All three forms of the enzyme were present, however, and the experiments thus demonstrate that

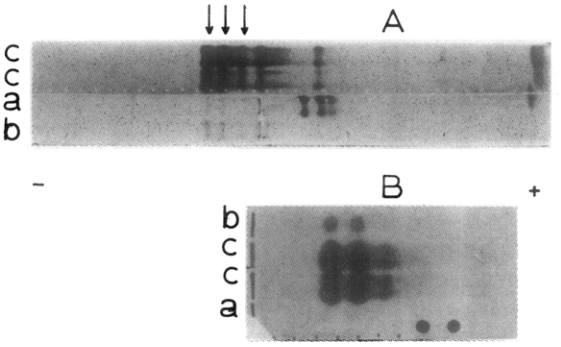

FIGURE 1: Electrophoretic pattern of rainbow trout enolase (indicated by arrows) on starch gel. (A) 0.025 M sodium borate buffer, pH 8.6; (a) Versene extract, (b) after heat step, and (c) after one ammonium sulfate fractionation. (B) 0.025 M Tris–phosphate buffer, pH 8.6; recrystallized enolase at (a) 0.5, (b) 5.0, and (c) 50 mg/ml of protein. In all cases 0.15 ml of a solution of protein dissolved in the corresponding electrophoresis buffer was applied to each slot in the gel.

TABLE I: Purification of Rainbow Trout Enolase.[a]

Fraction Step	Total Act. (units)[b]	Total Protein (g)	Sp Act. (units/g)	Recov (%)
Versene extract	1,510,000	61.0[c]	25	100
35–46% acetone precipitate	1,230,000	18.2[c]	68	82
Heat supernatant	1,010,000	3.46[c]	281	67
55–70% ammonium sulfate precipitate	630,000	1.85	340	42
Crystallization	367,000	1.04	353	24[d]
Recrystallization	220,000	0.58	390	15[b]
Sephadex G-100 chromatography[e]			410	

[a] The data are taken from a typical purification starting with 685 g of muscle. [b] The amount of enzyme which will give an absorbance change of 0.1/min at 240 mμ (Holt and Wold, 1961). [c] Based on the 280-mμ absorbance of the crude protein mixture and included primarily as a guide for future preparations. [d] The yield could be improved by pooling side fractions. [e] Only part of an enolase preparation was put on any one column.

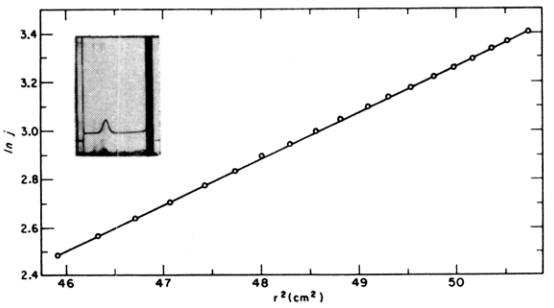

FIGURE 2: Test for the homogeneity of rainbow trout enolase with respect to weight-average molecular weight. The data are taken from an equilibrium run in the ultracentrifuge after 20 hr at 5° and 5325 rpm. The protein concentration was 5.3 mg/ml. *Insert:* schlieren pattern from a sedimentation velocity run after 80 min at 6.2° and 59,788 rpm. The protein concentration was 11 mg/ml and the bar angle was 75°. In both cases the solvent was 0.025 M Tris phosphate, 10^{-3} M magnesium sulfate buffer, pH 8.6.

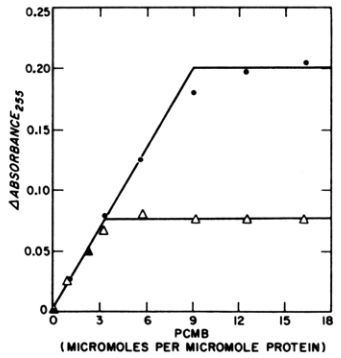

FIGURE 3: Titration of sulfhydryl groups in enolase. The titrations were carried out in 8 M urea–0.1 M sodium acetate buffer, pH 5.0. △—△, untreated enolase; ●——●, enolase reduced with borohydride in 8 M urea at pH 9.

the three enolases have very similar molecular weights.

Based on absorbance measurements of several samples dried to constant weight, the absorbance at 280 mμ of a 1-mg/ml solution of the pure enzyme was found to be 0.79 cm^{-1} with a 280/260 ratio of 2.00–2.05.

The amino acid analysis of rainbow trout enolase is given in Table II. The molecular weight of 91,800 based on 3 moles of tryptophan/mole of enzyme is in excellent agreement with the molecular weight obtained from the ultracentrifuge. Amino acid analyses of individual forms did not show any significant differences in their amino acid composition. The analyses were, however, conducted on small samples, and more careful analysis must be carried out in order to exclude small differences in the make-up of the three enolases.

TABLE II: The Amino Acid Content of Rainbow Trout Enolase.[a]

	Moles of Amino Acid/Mole of Tryptophan	Moles of Amino Acid/91,800 g of Protein (3 tryptophans/mole)	Residues/Mole (nearest integral)
Lysine	26.7	80.1	80
Histidine	6.9	20.7	21
Arginine	8.0	24.0	24
Aspartic acid	32.9	98.7	99
Threonine	11.1	33.3	33
Serine	16.5	49.3	49
Glutamic acid	29.6	88.8	89
Proline	10.4	31.2	31
Glycine	26.9	80.7	81
Alanine	30.7	92.1	92
Valine	18.5	55.5	56
Methionine	4.0	12.0	12
Isoleucine	18.5	55.5	56
Leucine	22.2	66.6	67
Tyrosine	7.4	22.1	22
Phenylalanine	9.4	28.1	28
Tryptophan	1.0	3.0	3
Half-cystine	2.6	7.8	8

[a] Average of two separate analyses, corrected for destruction during hydrolysis. Tryptophan and half-cystine were determined on single samples, the latter as cysteic acid after performic acid oxidation. The molecular weight calculated on the basis of this amino acid composition is 91,800.

The results of a direct titration of sulfhydryl groups in the enzyme are given in Figure 3, and show that the enzyme contains 3 moles of free SH groups and 3 moles of disulfide, the latter ones being released as SH groups after reduction with borohydride. The total of nine half-cysteines is in reasonable agreement with the value of 8 moles of cysteic acid found by amino acid analysis of the oxidized protein.

Some catalytic properties of the enzyme are given in Figures 4 and 5. All the determinations were carried out at constant ionic strength in 0.2 M KCl. As observed with other enolases (Malmström, 1961), KCl has a stimulatory effect on the rate of the enolase reaction. The optimal concentration is in the range of 0.2–0.3 M, as higher concentrations cause some inhibition. The pH optimum of the rainbow trout enolase is at 6.9. The absolute requirement for Mg^{2+} ions is clearly demonstrated, with an optimum Mg concentration near 10^{-3} M. From the data in Figure 5, the kinetic

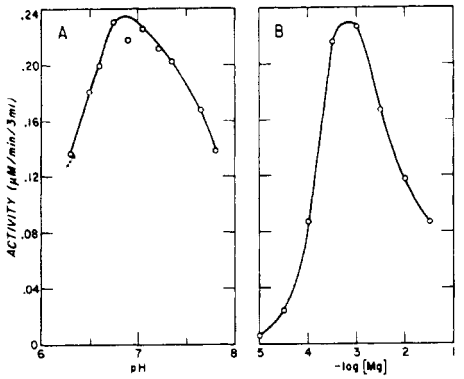

FIGURE 4: The effect of pH and magnesium ions on the rate of the enolase reaction. The observed rates in terms of optical density change were corrected for the effect of pH and Mg on the absorbance of enolpyruvic acid phosphate (Wold and Ballou, 1957). The buffer in both experiments was 0.05 M imidazole containing 0.2 M KCl. The substrate concentration was 10^{-3} M D-glyceric acid phosphate. The pH was held constant at 7.00 in B and the magnesium concentration constant at 10^{-3} M in A.

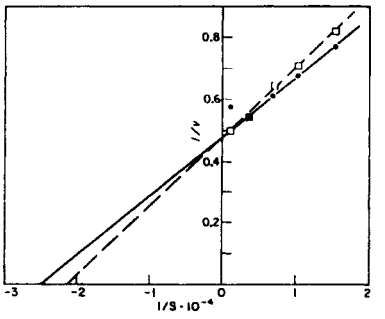

FIGURE 5: Lineweaver–Burk plots of the effect of substrate and of phosphate on the rate of the enolase reaction. The experiments were conducted in 0.05 M imidazole buffer, pH 6.95, in the presence of 0.2 M KCl and 10^{-3} M magnesium sulfate. ●——●, no inhibitor; □——□, in the presence of 10^{-2} M phosphate.

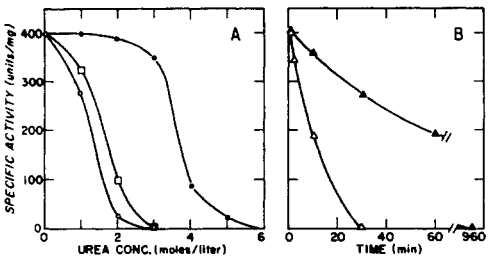

FIGURE 6: Urea denaturation of enolase at 25°. (A) The effect of urea concentration and time on the rate of denaturation of enolase. Enolase (0.06 mg/ml) was incubated in 0.05 M imidazole–10^{-3} M magnesium sulfate buffer, pH 7.9, in the presence of the urea concentrations indicated. The activity was determined by adding the enzyme directly to the assay medium after ●——●, □——□, and O——O, 1-, 30-, and 60-min incubation times, respectively. (B) The effect of substrate on the rate of denaturation in 3 M urea. The experimental conditions were the same as in A; △——△, no substrate; ▲——▲, 10^{-3} M D-glyceric acid 2-phosphate added.

constants were found to be $K_m = 4 \times 10^{-5}$ M and $V_{max} = 7900$ moles of substrate/min per mole of enzyme. Figure 5 also shows that phosphate ion inhibits the reaction competitively with a K_I of 5×10^{-3} M. The classical inhibition of enolase by fluoride, originally defined by Warburg and Christian (1942) as being due to the high affinity of yeast enolase for a magnesium–fluoride–phosphate complex, was also tested for the trout enzyme. The inhibition index was described by Warburg and Christian as

$$i = \frac{\text{residual activity}}{\text{inhibited activity}}[Mg^{2+}][HPO_4^{2-}][F^-]^2$$

and they found a value for i of 3.2×10^{-12} (M^4). A very similar value has been observed for rabbit muscle enolase (A. Holt, personal communication) and with the trout enzyme a value of 1.4×10^{-12} was obtained. When phosphate was omitted in the reaction mixture, the fluoride inhibition was eliminated.

It was felt of interest to establish the stability of the enzyme on exposure to a number of denaturing conditions. Thus the effect of pH on trout enolase was determined by incubating 0.5-mg/ml samples of enolase in different pH buffers at 4° and in the presence of 10^{-3} M Mg^{2+}. In the neutral pH range (pH 7.9 imidazole buffer or pH 8.6 Tris phosphate buffer) the enzyme could be kept at 4° for 1 week without any significant activity loss. In the alkaline range it was also quite stable, retaining full activity after exposure to pH 10.55 buffer for 8 hr. At pH 11 the half-life was approximately 24 hr and at pH 11.4, 30 min. In the acid range, however, the enzyme is quite sensitive, the half-life at pH 5 being about 2 hr and at pH 4 only a few minutes.

Trout enolase was also found to be quite sensitive to urea denaturation. Figure 6 shows that the enzyme is essentially completely inactivated in 1 hr by 2 M urea at 25°. In the presence of substrate, however, there is a significant decrease in the rate of urea denaturation (Figure 6).

It should be pointed out that all attempts to reverse the denaturation processes either by fast (dilution) or by slow (dialysis) removal of the denaturing agent were unsuccessful.

Discussion

Up to now the two best studied enolases are those from yeast and from rabbit muscle, and in Table III

TABLE III: Comparison of Some Properties of Three Enolases.

Property	Enzyme Source: Yeast	Rabbit Muscle	Trout Muscle
Molecular weight	67,000[a]	82,000[b]	91,000
Number of peptide chains/mole	1[a]	2[b]	—
Moles of SH/mole	0[a]	12[c]	3
Moles of SS/mole	0[a]	0[c]	3
Moles of N-terminal amino acid/mole	1 Ala[d]	2 N-acetylala[e]	—
Moles of C-terminal amino acid/mole	Leu[d]	2 Lys[e]	—
K_m[f] (moles/l.)	1×10^{-4}	6×10^{-5}	4×10^{-5}
V_{max}[f] (moles substrate/min mole of enzyme)	11,000	10,000	8,000
pH optimum	7.7[a]	6.9[a]	6.9
[Mg] optimum (moles/l.)	10^{-3}[g]	10^{-3}[g]	10^{-3}
F^- inhibition ($i = [V/V_0 - V][Mg^{2+}][HPO_4^{2-}][F^-]^2$)[h]	3.2×10^{-12}[h]	—	1.4×10^{-12}

[a] Malmström, 1961. [b] Winstead and Wold, 1965. [c] Malmström, 1962. [d] Malmström *et al.*, 1959. [e] Winstead and Wold, 1964. [f] Determined at the optimal pH, ionic strength, and Mg concentration for each enzyme. [g] Holt and Wold, 1961. [h] Warburg and Christian, 1942.

these enzymes are compared with trout muscle enolase with respect to some physical, chemical, and biochemical properties. Although there are differences in their catalytic properties, the similarities are much more striking. The kinetic constants are those determined under optimal conditions of pH and ionic strength, and while they show some quantitative differences, other features, such as the fluoride inhibition and the absolute requirement for divalent metal ions, are remarkably constant for three enzymes which differ so greatly in physical and chemical properties (Table III). To the first approximation, the data in Table III are consistent with a model of three quite different proteins having very similar active sites.

One interesting feature of the trout enzyme, which also expresses unique chemical properties, is its lability to acid, urea, and high salt, and the fact that the denaturation appears to be irreversible in all cases. Both rabbit muscle enolase and yeast enolase can be denatured in a number of ways, but as a general rule the denaturation is reversible (Malmström, 1962; Westhead, 1964; Winstead and Wold, 1965). The inactivation of trout enolase in ammonium sulfate in the presence of air is not understood. There are two lines of indirect evidence which tend to rule out SH oxidation as the cause of the inactivation. Thus the addition of 2-mercaptoethanol or dithiothreitol (Cleland, 1964) did not offer any protection against activity loss either in the absence or in the presence of air, and furthermore, a 100-fold molar excess of *p*-MB did not cause any loss of activity even after 6-hr incubation with the enzyme. Most enzymes in which SH groups play an important role would suffer a rapid loss of activity under these conditions. After deliberately denaturing the enzyme in ammonium sulfate in the presence of air, neither the fluorescence spectra nor the absorption spectra gave any indication that aromatic residues had been modified in the denaturation. Since the loss of activity under these conditions appears to be a common feature of several trout and salmon enolases (R. Ruth and D. M. Soja, personal communication), this reaction must be investigated further. The most likely cause of denaturation in all of these systems is that the trout enolase, like the other enolases studied, is built up of subunits, and that the irreversible loss of activity reflects a high rate of denaturation of the separate subunits as compared to the intact enzyme. Studies are in progress to investigate this possibility.

The comparative chemistry and biochemistry of the three forms of enolase in fish muscle (Tsuyuki and Wold, 1964) is still indeterminate. Until a satisfactory method has been developed to separate the enzymes in such a way that both catalytic and chemical parameters can be compared, both the chemical differences and the possible functional significance of the three forms remain obscure.

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Physical and Catalytic Properties of Tryptophanase from *Bacillus alvei**

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ABSTRACT: Tryptophanase from *Bacillus alvei* was resolved from its coenzyme, pyridoxal phosphate, by extended dialysis against Tris–EDTA buffer. Equilibrium dialysis of the enzyme against pyridoxal phosphate in potassium phosphate or Tris buffer indicated that 1 mole of coenzyme is bound/125,000 or 130,000 g of enzyme, in the respective buffers. Kinetic analysis of coenzyme binding suggests that two molecules of pyridoxal phosphate are required for full activity of each active site.

Dissociation constants calculated from the kinetic data for the two coenzyme molecules were 1.14 and 14.4 μM, respectively. A sedimentation constant of 10.8 S was calculated for the enzyme at infinite dilution in 0.01 M potassium phosphate, pH 7.0. The sedimentation constant decreases in buffers of higher ionic strengths. If resolved from coenzyme, the enzyme readily dissociates upon dilution in Tris–EDTA buffer, forming a particle with sedimentation constant about 5.4 S. The dissociated material can be fully reactivated by dialysis against potassium phosphate containing pyridoxal phosphate. The molecular weight of the enzyme in both the native and reconstituted form is approximately 220,000.

The authors have described the purification and some of the catalytic properties of tryptophanase from *Bacillus alvei* (Hoch *et al.*, 1966). The enzyme is constitutive and appears to participate in a regulatory role in the physiology of the organism (Hoch and DeMoss, 1965, 1966).

During additional sedimentation experiments, anomalous behavior of the protein was observed. In this report, we present data concerning the enzyme–coenzyme complex and some properties of the apoenzyme.

Materials and Methods

Tryptophanase. Tryptophanase was purified as previously described from extracts of *B. alvei* (Hoch *et al.*, 1966). The purified preparation was subjected to chromatography on Sephadex G-200 to remove a small amount of contaminating nucleic acid. The 280/260 mμ absorbancy ratio of the final preparation was 1.75. Tryptophanase activity was assayed as previously described (Hoch and DeMoss, 1965). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Pyridoxal Phosphate. Pyridoxal phosphate[1] was determined with phenylhydrazine as described by Wada and Snell (1961). Protein was precipitated from the contents of the dialysis bags by the addition of H_2SO_4 before determination of the PLP content (Dempsey and Snell, 1963).

Sedimentation. Sedimentation experiments were performed in a Spinco Model E ultracentrifuge. The density of all buffers was assumed to be the same as the density of water at the temperatures used. Sedimentation velocity experiments were conducted at 59,780 rpm in the standard 4° sector cell. Sedimentation equilibrium determinations were performed at 20° in a double-sector cell at 7257 rpm. Speed was determined from the

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[1] Abbreviation used: PLP, pyridoxal 5-phosphate.