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Thyroidal Phenylpyruvate Tautomerase

ISOLATION AND CHARACTERIZATION

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SUMMARY

Hog thyroid tautomerase (EC 5.3.2.1, phenylpyruvate keto-enol isomerase), an enzyme which is believed to play a role in the biosynthesis of thyroxine, has been purified over 1000-fold.

The purification procedure involves a heat step at 70°, gel filtration on Sephadex G-100, ion exchange chromatography on carboxymethyl Sephadex C-50, and, finally, gel filtration on Sephadex G-50.

The enzyme is homogeneous by the following criteria. It gives a single peak in sedimentation velocity analysis and shows a single band on disc polyacrylamide gel and starch gel electrophoresis, and the sedimentation equilibrium plot is linear, even at the lowest protein concentrations.

The pH optimum of the purified tautomerase is 6.2. Its molecular weight is 44,000. The amino acid composition of the enzyme, as well as some of its physico-chemical properties, are presented. The enzyme is able to substitute for borate ions in the coupling reaction of thyroxine synthesis.

The mechanism of thyroxine biosynthesis is still partly unknown. It is known that diiodotyrosine *in vivo* is a precursor of the thyroid hormone (1). On this basis some chemical models have been proposed and their mechanisms studied in detail (2-4). From the studies on one of these models, the coupling of diiodotyrosine with its keto acid derivative, 3,5-diiodohydroxyphenylpyruvic acid (3, 4), a possible pathway of thyroxine biosynthesis has been proposed (5). The physiological significance of this pathway is supported by the presence in the thyroid gland of enzymes that catalyze the individual steps of the pathway. These enzymes are diiodotyrosine transaminase, diiodohydroxyphenylpyruvic acid tautomerase, and peroxidase (5).

The isolation of two of these enzymes from the thyroid gland, the transaminase (6) and the peroxidase (7), has been reported. In this paper we describe the isolation and properties of hog thyroid tautomerase (EC 5.3.2.1, phenylpyruvate keto-enol isomerase).

EXPERIMENTAL PROCEDURE

Materials

Hog thyroid glands, obtained from a local slaughterhouse, were collected over a period of 1 month and stored frozen within 1 hour from the death of the animal.

The substrate hydroxyphenylpyruvic acid was purchased from Mann. 3,5-Diiodohydroxyphenylpyruvate and phenylpyruvate were obtained from Calbiochem; crystalline bovine serum albumin, from Armour, and trypsin and pig heart mitochondrial malate dehydrogenase from Boehringer Mannheim. Sephadex G-50, Sephadex G-100, and carboxymethyl Sephadex C-50 were products of Pharmacia. Sephadex G-100 particles were sieved in the swollen state through stainless steel sieves and the particles between 100 and 200 mesh were used. Carboxymethyl Sephadex C-50, and DEAE-cellulose (Whatman DE-22) were treated as recommended by the manufacturers.

Methods

Enzyme Assay—For convenience, hydroxyphenylpyruvic acid was routinely used as substrate throughout the purification procedure. The iodo-keto acid, in fact, is a very unstable compound (3, 4). The assay was carried out by the method of Knox (8), measuring the increase in absorbance at 330 mμ caused by the formation of the enol-borate complex of hydroxyphenylpyruvate. A molar extinction coefficient of 6330[1] was used. The reaction mixture contained 4.0 μmoles of hydroxyphenylpyruvate, 10 μmoles of acetate, 3.2 μmoles of sodium azide, 1.5 mmoles of boric acid, pH 6.2, and enzyme at appropriate concentration, in a total volume of 3.2 ml. The reaction was started by the addition of the substrate. A blank was prepared in the same way except that substrate was omitted. One enzymatic unit was defined as the amount of enzyme catalyzing the keto-enol tautomerism of 1 mμmole of substrate per min.

Protein Determination—For calculation of enzymic activities, proteins were determined according to the biuret method (9), the method of Lowry *et al.* (10), and the microbiuret method (11), with crystallized bovine serum albumin as a standard. The protein concentration during separation procedures was followed by the measurement of the absorbance at 280 mμ.

Linear Sucrose Density Gradient Centrifugation—Preparation, centrifugation, and collection of sucrose density gradients were carried out as described by Martin and Ames (12). The linear gradient (5 to 20%), prepared in 0.1 M phosphate buffer, pH 6.2, was run in the Beckman-Spinco model L2-65 centrifuge at 4° in the SW 65 rotor at 63,000 rpm for 15 hours. The rotor, protein sample, sucrose gradient, and the centrifuge itself were equilibrated at 4° prior to the run.

Polyacrylamide Disc Gel Electrophoresis—Disc gel electrophoresis in 7% polyacrylamide at pH 9.5 was performed according to

[1] F. Blasi, unpublished observations.

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the method of Davis (13). This technique was used to monitor the purification at each step of the isolation procedure. Reagents were supplied by Canalco. Gels were stained for proteins with 0.2% Amido black (Allied Chemical Company) in 5% acetic acid, and destained with 7% acetic acid. Electrophoresis was run for 1 hour at 4° at +4 mA per tube.

Starch Gel Electrophoresis—Starch gel, gel buffer, and electrode buffer were prepared according to the method of Barrett, Friesen, and Astwood (14). The electrophoresis was carried out for 90 min at 32 mA and 400 volts. Staining and destaining were performed as described under "Polyacrylamide Disc Gel Electrophoresis."

Amino Acid Analyses—Samples of protein (approximately 1 mg) were extensively dialyzed against distilled water and then lyophilized. Hydrolyses were carried out with constantly boiling HCl (1 mg of protein per ml) at 106° for 22 to 96 hours. Following hydrolysis, the samples were dried over NaOH in vacuum, and amino acid analyses were carried out in a Spinco amino acid analyzer, model 120 B.

The number of tryptophan residues was estimated according to the method of Edelhoch (15) in 6 M guanidine-HCl.

Analytical Ultracentrifugation—Sedimentation velocity and equilibrium sedimentation studies were carried out in a Spinco model E analytical ultracentrifuge. The sedimentation coefficient was determined by standard procedures, using a double sector standard cell, at 56,700 rpm. Photographs were taken every 16 min.

The molecular weight was measured by the meniscus depletion method of Yphantis (16) at 39,733 and 35,600 rpm, using Rayleigh interference optics and a double sector standard cell. The attainment of equilibrium was experimentally verified. Measurement of fringe displacement was carried out by means of a Nikon two-dimensional microcomparator.

From the observed sedimentation ceofficient and molecular weight values, a diffusion constant was calculated according to the Svedberg equation.

$$M = \frac{R \cdot T \cdot Sobs}{D(1 - \bar{v}\rho)}$$

For this calculation and for calculation of the molecular weight from the sedimentation equilibrium studies, a partial specific volume $\bar{v}$ of 0.73 was used, which was calculated from the amino acid analysis according to the method of Cohn and Edsall (17).

A frictional ratio, f/f_0, was calculated from the molecular weight, sedimentation coefficient, and diffusion constant (18). From the value of the frictional ratio an axial ratio was determined as described by Tanford (19).

For all the described studies, the protein was extensively dialyzed against 0.1 M phosphate buffer, pH 7.4, containing 0.14 M potassium chloride.

Absorption Spectra—Spectrophotometric analyses were carried out in a Beckman DK-2 recording spectrophotometer in quartz cells of 1.0-cm light path.

Coupling Reaction of Thyroxine Synthesis—Thyroxine synthesis was carried out as previously described (4). The reaction mixture contained, in a total volume of 2.0 ml: 7.0 μmoles of ^{125}I-diiodotyrosine (10 μCi per μmole), 7.0 μmoles of diiodohydroxyphenylpyruvic acid, and 400 μmoles of buffer (see Table IV). In some experiments 5 to 30 μg of purified hog thyroid tautomerase, dissolved in 25 to 50 μl of 0.1 M phosphate buffer, pH 7.4, containing 0.14 M potassium chloride, were added to the reaction mixture. The reaction mixture was flushed 2 min with oxygen and then left in air for an additional 18 min with stirring. The reaction was carried out at room temperature. At the end of the incubation period, 0.1 ml of 2 N NaOH was added. The synthesized thyroxine was separated from the other reactants by chromatography on Sephadex G-25. The column (1.5 × 21 cm) was equilibrated with 0.02 N NaOH and eluted with the same solvent (20). The amount of ^{125}I-thyroxine synthesized was calculated on the basis of the initial ^{125}I-diiodotyrosine radioactivity.

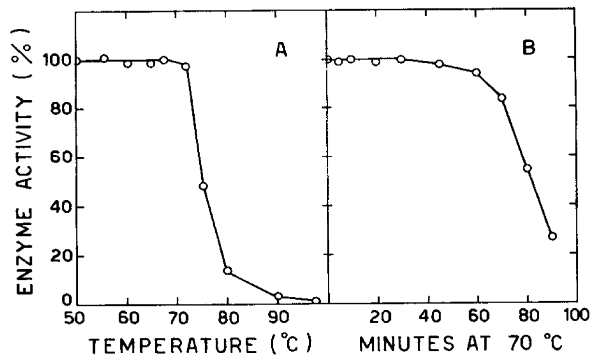

FIG. 1. Heat stability of hog thyroid tautomerase. *A*, the hog thyroid extract (Step I of the Purification Procedure) was incubated at various temperatures for 5 min in standard buffer at a protein concentration of 5 mg per ml. *B*, the enzyme preparation (same as in *A*) was incubated for various periods of time at 70° in standard buffer at a protein concentration of 5 mg per ml.

RESULTS

Stability

The hog thyroid tautomerase has been found to be rather stable toward both time and temperature. Enzyme activity is only slightly decreased (5 to 8% in 20 days) when crude thyroid extract is stored at 4° at a protein concentration of 5 or 50 mg per ml. Storage for 6 weeks at −20° did not result in any significant loss of activity.

No decrease in activity was found when the crude extract was heated for 5 min at various temperatures up to 72° (Fig. 1*A*). The thyroid extract can be exposed to a temperature of 70° for as long as 30 min with no significant loss of activity. Less than 30% activity, however, is recovered after exposure of the extract to 70° for 90 min (Fig. 1*B*). Although not shown in the figure, the specific activity increased up to 40 min, and then remained constant. An increase in specific activity of about 5-fold was obtained after 25 min at 70°.

Purification Procedure

Homogenization (*Step I*)—All manipulations were carried out at 4° unless otherwise specified. Hog thyroid glands were freed from fat and connective tissue, weighed (3 kg), cut in small pieces, and homogenized with 6.0 liters of 0.1 M phosphate buffer, pH 7.4, containing 0.14 M potassium chloride, hereafter referred to as the standard buffer. The homogenization was carried out for 2 min in a Waring Blendor. The homogenate was centrifuged at 700 × *g*; the thick upper fat layer was removed with a porcelain spatula, the sediment was discarded, and the supernatant fraction was recentrifuged at 20,000 × *g*. After two washings of the sediment, the combined supernatants were filtered through

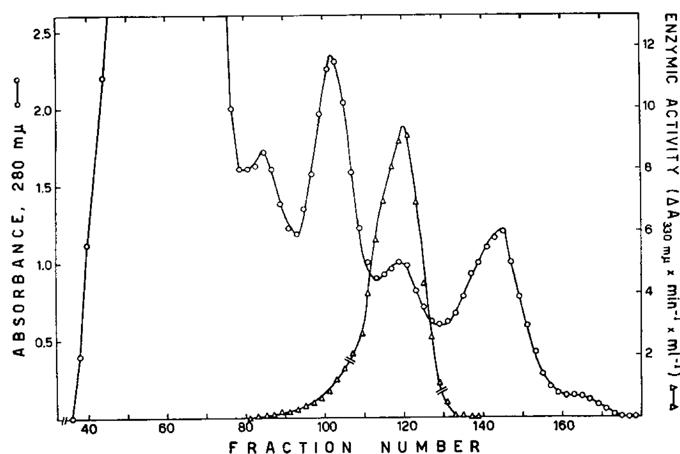

FIG. 2. Elution pattern of hog thyroid tautomerase activity on a Sephadex G-100 column (7 × 105 cm) equilibrated with the standard buffer containing 1 mM NaN_3. The sample applied to the column had a volume of 71 ml and a protein concentration of 78 mg per ml.

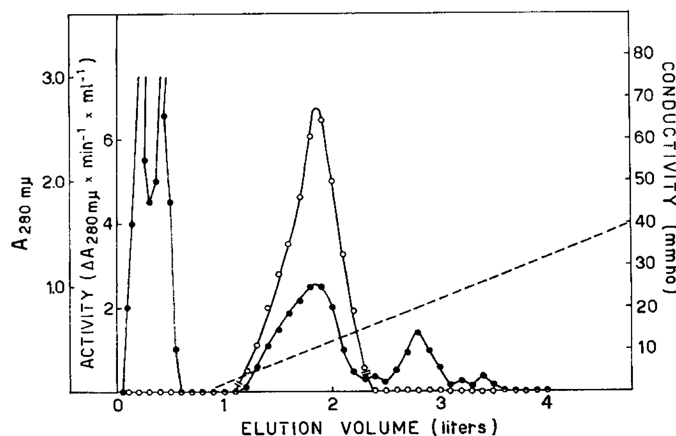

FIG. 3. Elution pattern of hog thyroid tautomerase on carboxymethyl-Sephadex C-50 column (3 × 32 cm) equilibrated with 0.01 M phosphate buffer, pH 6.0, containing 1 mM NaN_3. Enzyme activity was eluted with a linear gradient of KCl (0 to 1.0 M, 1.5 liters each) in 0.01 M phosphate buffer, pH 6.0, containing 1 mM NaN_3. O——O, enzyme activity ($\Delta A_{330m\mu} \times min^{-1} \times ml^{-1}$); ●——●, absorbance at 280 mμ; – – –, conductivity.

three layers of cheesecloth and then recentrifuged at 67,000 × *g* for 30 min. The supernatant was again filtered through three layers of cheesecloth, and used in the next step.

DEAE-cellulose (*Step II*)—Two DEAE-cellulose columns, 7 × 60 cm, 2.25-liter bed volume, equilibrated with 0.01 M phosphate buffer, pH 6.6, were prepared. The enzyme preparation (4.62 liters, 60 mg of protein per ml), dialyzed against 0.01 M phosphate buffer, pH 6.6, was divided into two batches of equal volume, which were passed through the columns. These were then washed with 1 volume of buffer. This step was employed in order to eliminate the bulk of thyroglobulin, which constitutes the majority of thyroidal protein and which remains adsorbed to the resin (21). The enzymic activity, on the contrary, was completely eluted (87% recovery), and a 7-fold increase in specific activity was obtained.

Heat Treatment (*Step III*)—The eluates of the DEAE-cellulose columns (7.14 liters, 11.5 mg of protein per ml) were fractionated with ammonium sulfate (0.465 g per ml). The resulting suspension was left standing overnight. The precipitate was collected by centrifugation at 20,000 × *g* for 20 min, redissolved in standard buffer, and dialyzed extensively against several changes of this buffer. In the preparation described in this report, the addition of the salt lowered the pH of the solution below pH 5.0; this might be responsible for the loss of about 20% of the enzymic activity. The dialyzed material was rapidly brought to 70° in 100-ml batches (75 mg of protein per ml) and left at this temperature for 25 min, then rapidly cooled in ice and centrifuged. The sediment was discarded. A 5-fold purification was achieved by this step with a recovery of almost 100% of the activity.

Gel Filtration (*Step IV*)—The supernatant of the heat-treated material was concentrated by precipitation with solid ammonium sulfate (0.465 g per ml), controlling the pH with a pH meter and keeping it constant at 7.4 by adding a few drops of ammonium hydroxide when necessary. The precipitated material was collected by centrifugation, redissolved, and extensively dialyzed against standard buffer. The dialyzed sample (142 ml, 78 mg of protein per ml) was layered onto two Sephadex G-100 columns, 7 × 105 cm, 4-liter bed volume, which were equilibrated with the standard buffer containing 1 mM sodium azide. This compound, which has been shown to have no effect on thyroidal tautomerase activity (5), was used as a bacteriostatic agent. The flow rate was kept close to 1 ml per cm² of column cross section per hour. The elution profile of the two columns was identical and the pattern of one of them is shown in Fig. 2. By this step the specific activity was increased 8-fold. The pooled fractions, containing 71% of the activity recovered after the first ammonium sulfate precipitation, were dialyzed against 0.01 M phosphate buffer, pH 6.0, containing 1 mM sodium azide.

Ion Exchange Chromatography (*Step V*)—The dialyzed material (422 ml, 1.85 mg of protein per ml) was applied to a carboxymethyl Sephadex C-50 column, 3 × 32 cm, 300-ml bed volume, in 0.01 M phosphate buffer, pH 6.0, containing 1 mM sodium azide. The column was washed with 3 volumes of this buffer. The enzyme activity was retained on the column and was eluted with a linear gradient (0 to 1 M) of potassium chloride in the same buffer. The elution profile is shown in Fig. 3. By this step, approximately 68% recovery was obtained, with more than 3-fold increase in specific activity.

Gel Filtration (*Step VI*)—The pooled fractions (see Fig. 3) containing the enzymic activity (990 ml, 0.12 mg of protein per ml) were dialyzed against standard buffer, concentrated about 10-fold in a flash evaporator at 30° and precipitated with ammonium sulfate (0.465 g per ml) at a constant pH of 7.4. The precipitate was collected by centrifugation, dissolved in a minimal amount of standard buffer, and dialyzed. A sucrose density gradient (5 to 20%) ultracentrifugation of the dialyzed material revealed that the enzymic activity was contaminated (10 to 15%) by a lower molecular weight material. In order to eliminate this contaminant, the enzyme preparation (3 ml, 25 mg of protein of ml) was subjected to gel filtration through a column of Sephadex G-50, 1.5 × 105 cm, 185-ml bed volume. A single symmetrical peak of enzyme activity was eluted from the column, coincident with the main protein peak, and well separated from minute amounts of retarded inactive material. The pooled fractions, having a specific activity 1.3 times higher than in the previous step, were concentrated about 2-fold in a flash evaporator in order to obtain a protein concentration (~1.5 mg per ml) suitable for ammonium sulfate precipitation. The sediment of the ammonium sulfate

precipitation was collected by centrifugation, redissolved, and dialyzed against standard buffer. In the last two concentration steps, employing the flash evaporator and the ammonium sulfate precipitation, an insoluble precipitate appeared and 30 to 40% of the enzyme activity and protein was lost. The active material obtained at this step is hereafter referred to as the purified enzyme. The recovery and the degree of purification achieved at each step are given in Table I.

Criteria of Purity

Disc and Starch Gel Electrophoresis—When subjected to disc gel electrophoresis in polyacrylamide at pH 9.5, 100 μg of purified enzyme showed a single band (Fig. 4). Starch gel electrophoresis (380 μg of purified enzyme) revealed a single band moving toward the cathode (Fig. 5).

Ultracentrifugal Pattern—Fig. 6 shows the ultracentrifugal schlieren pattern of a solution of purified enzyme in the standard buffer at a protein concentration of 6 mg per ml. The enzyme showed only a single peak with a sedimentation coefficient of 2.7 S. In a separate experiment, carried out at a concentration of 4 mg per ml, a sedimentation coefficient of 2.6 S was obtained.

Enzyme Properties

pH Optimum—The pH dependence of the reaction rate with hydroxyphenylpyruvate as a substrate is shown in Fig. 7. The maximal rate is reached between pH 6.0 and 6.2. The values reported in the figure, do not represent V_{max} (which cannot be obtained because of the high extinction coefficient of the enol-borate complex) and are not corrected for the nonenzymic reaction, which is not negligible above pH 7.0 (22). This might explain the shoulder on the alkaline wing of the pH activity curve.

Kinetic Parameters—Hydroxyphenylpyruvate and its diiodinated derivative have a K_m of 1.2×10^{-3} M, whereas phenylpyruvate has a K_m of 8×10^{-3} M.

TABLE I

Purification of hog thyroid tautomerase

Purification step	Protein	Total activity	Specific activity	Yield
	g	*e.u.* $\times 10^{-4}$	*e.u./mg*	%
I. Supernatant of 67,000 × *g*	555.0	1,225	2.2	95.0
II. DEAE-cellulose eluate	75.6	1,073	14.1	83.0
III. Heat treatment	11.1	858	77.3	66.5
IV. Gel filtration, Sephadex G-100	0.78	412	541.2	31.9
V. Ion exchange, Cm-Sephadex C-50	0.15	280	1,830.0	21.7
VI. Gel filtration, Sephadex G-50	0.055	126	2,300.0	9.8

Hydrodynamic Properties—The molecular weight of the enzyme has been determined by the meniscus depletion method of Yphantis (16). A straight line was obtained by plotting log C against X^2 (Fig. 8), thus further confirming the high degree of homogeneity of the enzyme preparation. From the slope of this straight line a molecular weight of 44,000 has been calculated. In preliminary studies the molecular weight of the enzyme was determined on less pure preparations by gel filtration and sucrose density gradient ultracentrifugation. In the first case, trypsin, bovine serum albumin, and pig heart malic dehydrogenase were used as markers (see Reference 23). In the second case, bovine serum albumin was used as a marker. Molecular weights of 39,000 and 44,000 were obtained by gel filtration and by sucrose density gradient centrifugation, respectively.

Some further physico-chemical parameters, calculated from the molecular weight and the sedimentation coefficient, are listed in Table II.

Absorption Spectra and Extinction Coefficient—An approximate extinction coefficient at 280 mμ for the purified enzyme was determined by measuring the absorbance at 280 mμ in 0.1 M phos-

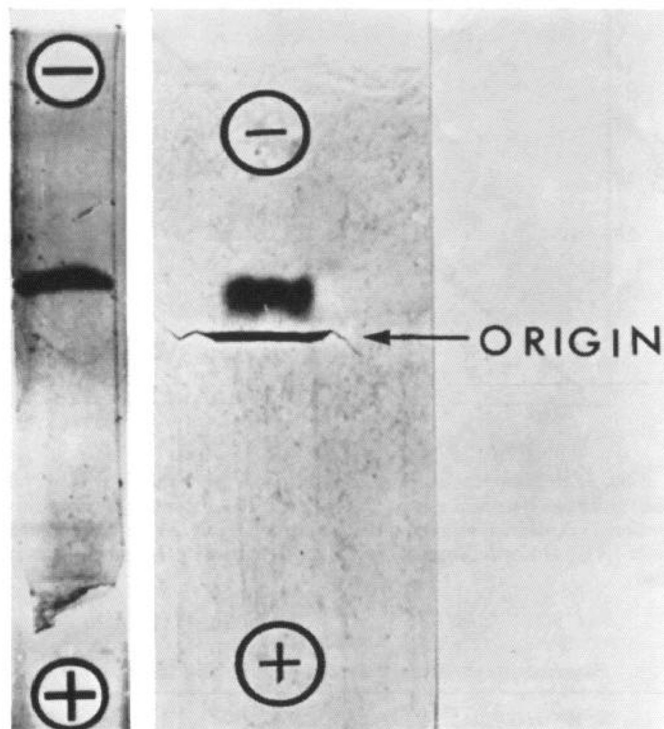

FIG. 4 (*left*). Polyacrylamide disc gel electrophoresis of 100 μg of purified hog thyroid tautomerase.

FIG. 5 (*right*). Starch gel electrophoresis of 380 μg of purified hog thyroid tautomerase.

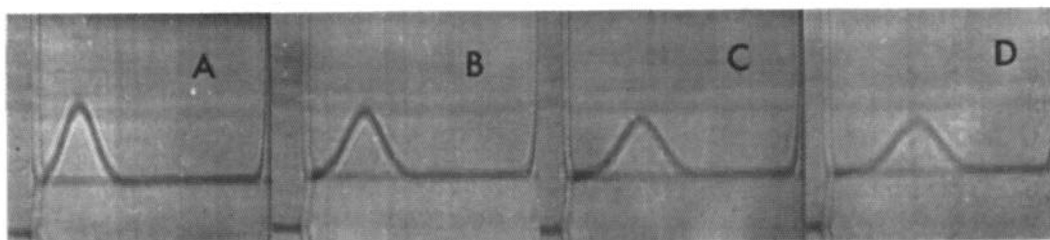

FIG. 6. Sedimentation velocity pattern of purified hog thyroid tautomerase (protein concentration, 6 mg per ml in standard buffer). The pictures were taken at a bar angle of 60° at the following times after reaching top speed: *A*, 32; *B*, 48; *C*, 64; *D*, 80 min.

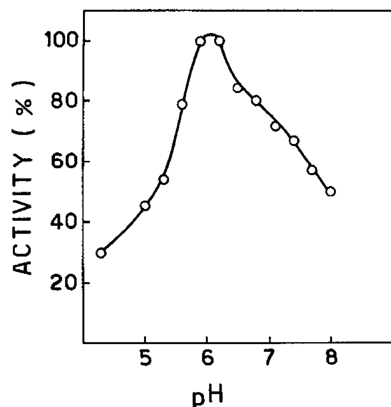

FIG. 7. pH Dependence of hog thyroid tautomerase activity assayed with hydroxyphenylpyruvate as substrate.

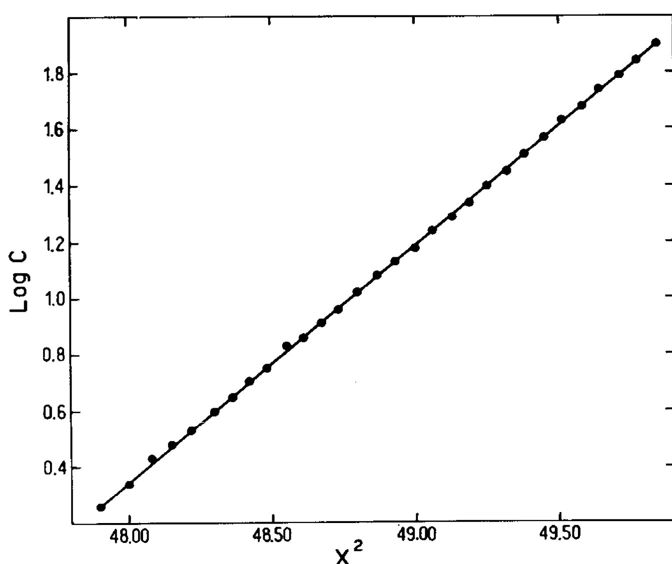

FIG. 8. Sedimentation equilibrium of purified hog thyroid tautomerase (protein concentration, 0.29 mg per ml in standard buffer). *Abscissa*, square of the distance from the center of rotation (X^2); *ordinate*, logarithm of the fringe displacement parameter.

TABLE II

Physical properties of purified hog thyroid tautomerase

$s_{20,w}$, $\times 10^{13}$ sec^{-1}	2.7
Molecular weight	44,000
$D_{20,w}$, $\times 10^7$ cm^2 sec^{-1} (calculated)	7.5
Partial specific volume ($\bar{v}$)[a]	0.73
Frictional ratio (f/f_0)	1.25
Axial ratio (a/b)	5.0

[a] Calculated from the amino acid composition according to the method of Cohn and Edsall (17).

phate buffer, pH 6.2. The protein concentration was measured with the microbiuret method (11), and checked with the results of the amino acid analysis. A value of 13.5 for $\epsilon^{1\%}$ has been obtained.

In Fig. 9 the absorption spectra of the purified hog thyroid tautomerase at neutral pH in phosphate buffer, and at both neutral and alkaline pH in 6 M guanidine-HCl, are depicted.

TABLE III

Amino acid composition of hog thyroid tautomerase

Amino acid	Time of hydrolysis				Average value[a]	No. of residues per 44,000[b]
	22 hrs	48 hrs	72 hrs	96 hrs		
	μmoles amino acid/100 mg protein					
Lysine	23.0	22.6	25.2	24.7	23.8	10
Histidine	13.9	14.9	16.1	14.7	14.9	7
Ammonia	78.8	100.4	116.0	129.5	115.5	50
Arginine	32.2	37.1	37.5	37.6	37.4	16
Aspartic acid	63.7	70.5	77.5	77.5	75.1	33
Threonine	17.8	19.3	19.8	18.7	18.9	8
Serine	51.6	50.7	49.7	43.6	52.4[c]	22
Glutamic acid	58.5	65.1	68.0	68.7	67.3	29
Proline	43.5	45.1	51.2	51.2	47.8	21
Glycine	58.5	64.9	70.5	71.0	69.0	30
Alanine	61.4	67.0	74.3	74.5	72.0	31
Half-cystine	16.5	18.9	21.9	19.2	19.0	8
Valine	48.6	58.2	63.2	64.0	61.6	27
Methionine	22.6	25.0	26.6	23.7	24.5	11
Isoleucine	33.1	37.1	40.5	39.5	39.0	17
Leucine	64.4	70.9	79.0	78.0	76.0	33
Tyrosine	29.4	29.8	32.2	30.4	30.4	13
Phenylalanine	26.0	27.7	30.3	30.1	28.5	12
Tryptophan[d]						4

[a] The full yields of ammonia, aspartic acid, glutamic acid, glycine, valine, isoleucine, and leucine were not obtained before 48 hours of hydrolysis and therefore the 22-hour determinations were not used in the calculations. The data for each time of hydrolysis represent the average of the analyses of two separate hydrolysates.

[b] Approximated to the nearest integer.

[c] Extrapolated to zero hour hydrolysis.

[d] Measured according to the method of Edelhoch (15).

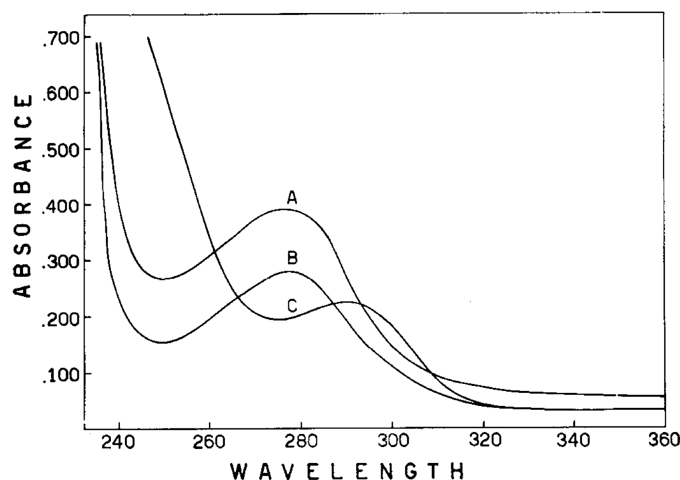

FIG. 9. Absorption spectra of purified hog thyroid tautomerase. *A*, 0.28 mg per ml in 0.1 M phosphate buffer, pH 7.0; *B*, 0.2 mg per ml in 6 M guanidine-HCl, pH 7.0; *C*, 0.2 mg per ml in 6 M guanidine-HCl, pH 12.

Amino Acid Composition—Table III lists the amino acid composition of the purified enzyme estimated from the duplicate results obtained at four different times of hydrolysis.

The number of tryptophan residues, calculated according to the method of Edelhoch (15), is 4.2, assuming a molecular weight of 44,000.

TABLE IV

Role of borate ions and thyroid tautomerase in coupling reaction of thyroxine synthesis

The reaction was carried out for 20 min at room temperature, incubating 7.0 μmoles each of ^{125}I-diiodotyrosine (10 μCi per μmole) and of DIHP. The reaction mixture was then analyzed by column chromatography and the yield of thyroxine calculated as the percentage of ^{125}I-radioactivity converted to thyroxine. For details see "Methods."

Buffer[a]	Addition	Yield of thyroxine
		μmoles
0.2 M Na_3BO_3-HCl	None	1.21
0.2 M phosphate	None	0.42
0.2 M Tris-HCl	None	0.31
0.2 M phosphate	Tautomerase (5 μg)	0.65
0.2 M phosphate	Tautomerase (10 μg)	0.98
0.2 M phosphate	Tautomerase (15 μg)	1.04
0.2 M phosphate	Tautomerase (30 μg)	1.31

[a] All buffers are pH 7.8.

Role of Tautomerase in Coupling Reaction of Thyroxine Synthesis—As shown in Table IV, the yield in thyroxine by the nonenzymic coupling of diiodotyrosine and DIHP[2] depends on the presence of borate ions. This confirms our previous data (4). However, the yield of thyroxine in the absence of borate ions can be raised to the level obtained in the presence of this ion by running the reaction in the presence of purified hog thyroid tautomerase.

The equilibrium ratio between the enol and keto forms of diiodohydroxyphenylpyruvic acid is less than 0.1 in phosphate buffer, whereas it is about 1.0 in the presence of borate (3); this explains the higher efficiency of the synthesis of thyroxine in the presence of the borate ions. It is therefore very interesting to find that the tautomerase can act as efficiently as the borate ions in the synthesis of thyroxine. This is probably explained by the higher initial rate of keto → enol conversion observed in the presence of enzyme (5).

DISCUSSION

The mechanism of the model reaction of thyroxine biosynthesis, the coupling of diiodotyrosine with DIHP, has been elucidated only quite recently (3, 4). It has been shown that the true partner of diiodotyrosine in thyroxine synthesis is not the keto acid itself, but an oxidized form of it, that is, the DIHP hydroperoxide. The formation of the hydroperoxide can occur, however, only if the keto acid is in its enol tautomeric form (3, 4).

Thyroid homogenate catalyzes the synthesis *in vitro* of thyroxine from diiodotyrosine through the intermediate formation of DIHP (5).

On the basis of these results, and also of the presence of the keto acid in the extracts of thyroid glands (24), a hypothesis has been advanced according to which thyroxine synthesis *in vivo* occurs by the coupling of DIHP hydroperoxide with a diiodotyrosine residue in thyroglobulin (5).

As already reported, the thyroid gland contains a soluble tautomerase which catalyzes the keto-enol tautomerization of DIHP, as well as of hydroxyphenylpyruvate and phenylpyruvate (5). The isolation of this enzyme, as well as of the other enzymes (6, 7) which are believed to be involved in thyroxine biosynthesis (5) is a necessary step in verifying the proposed pathway. In the present paper a tautomerase has been purified from the hog thyroid gland. The enzyme preparation appears to be homogeneous by the following criteria: polyacrylamide disc gel and starch gel electrophoresis, sedimentation velocity, and the linearity of the sedimentation equilibrium plot.

The amino acid composition and some physico-chemical parameters (Tables II and III) of the purified enzyme have been determined. A molecular weight of 44,000 has been obtained.

The phenylpyruvate tautomerase enzymes from lamb and pig kidney have very broad pH optima in the crude extract (22). The pH activity curve of the lamb kidney enzyme becomes sharper after partial purification, the optimum being between pH 4 and 5 (25). In contrast, the pH activity profile of the thyroid enzyme does not change during purification.[1] This suggests the possibility that only one tautomerase activity is present in the thyroid gland. The shoulder in the alkaline limb of the pH activity curve can be readily explained by the slow rate of spontaneous tautomerization of the keto acid which occurs at neutral and alkaline pH (22).

As shown in Table IV, thyroid tautomerase is able to substitute borate in the coupling reaction of thyroxine synthesis. This supports the view that a tautomerase plays a role in the biosynthesis of thyroid hormones (5). In fact, the second step of the pathway of thyroxine biosynthesis, proposed on the basis of studies *in vitro* (5), is the enol-keto isomerization of DIHP. In aqueous solutions DIHP is found almost quantitatively in its ketonic tautomeric form, except in the presence of high concentration of borate ions (3). The presence of this enzyme in the thyroid gland is in accord with the finding that only the enol form of DIHP is able to form DIHP hydroperoxide (3, 4), which is required for the subsequent formation of thyroxine. The fact that the tautomerase can substitute for borate ions, rules out the possibility that the enol-borate complex, rather than the enol form of DIHP is the substrate for the subsequent step of the pathway.

The reaction employed in the experiments in Table IV takes place nonenzymically, provided that oxygen is present in the reaction mixture (4). The presence of borate ions increases the yield of thyroxine by providing a rate of keto-enol tautomerism fast enough to ensure maximum yield of thyroxine under the conditions adopted, within the 20 min of incubation. In fact only about 20% of DIHP couples with diiodotyrosine to form thyroxine (4). This is most likely due to the degradation of DIPH during the oxidation to DIHP hydroperoxide (3, 4, 26).

The ability of tautomerase to substitute for borate ions is caused by the much faster rate of tautomerism with respect to the nonenzymic reaction (requiring borate).[1] This is, however, not evident from the data of Table IV because the rate of thyroxine synthesis in that system is limited by the rate of oxidation of DIHP (4).

It has been reported (5) that the tautomerase activity of the thyroid gland is in excess to that of diiodotyrosine transaminase, the enzyme that probably catalyzes the synthesis of the iodoketo acid. The low level of diiodotyrosine transaminase suggests that the reaction catalyzed by this enzyme is probably rate-limiting with respect to the rate of keto-enol tautomerism.

[2] The abbreviation used is: DIHP, 4-hydroxy-3,5-diiodophenylpyruvate.

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