The Purification and Properties of L-Histidine-2-Oxoglutarate Aminotransferase from *Pseudomonas testosteroni*

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1. Inducible L-histidine-2-oxoglutarate aminotransferase was purified some 170-fold from extracts of *Pseudomonas testosteroni*. 2. The preparation showed only one major component after electrophoresis on polyacrylamide gels, though additional minor bands were observed when samples concentrated on a DEAE-cellulose column were used. 3. The molecular weight of the enzyme was found to be approx. 70000 by chromatography on Sephadex G-200. 4. The purification scheme produced enzyme that was inactive in the absence of pyridoxal 5'-phosphate. 5. The equilibrium constant for the reaction L-histidine+2-oxoglutarate $\rightleftharpoons$ imidazolylpyruvate+L-glutamate was 0.49. 6. The reaction mechanism was Ping Pong. 7. The enzyme was shown to have only low activity towards aromatic amino acids and was highly specific for 2-oxoglutarate.

Pseudomonas testosteroni is capable of growth on imidazolyl-L-lactate as sole carbon source (Hassall, 1966). The pathway for the degradation of this compound has been established as consisting of an initial oxidation to imidazolylpyruvate, followed by a glutamate-dependent aminotransferase reaction to give L-histidine, which is then degraded in five enzymic steps to L-glutamate (Coote & Hassall, 1973a). All the enzymes of the pathway are inducible, and the ability to degrade imidazolyl-lactate is retained in cells grown on histidine, urocanate or imidazolylpropionate. The activities of histidine ammonia-lyase (EC 4.3.1.3), histidine-2-oxoglutarate aminotransferase (EC 2.6.1.38) and urocanase (EC 4.2.1.49) are consistent with these enzymes being subject to co-ordinate control under most growth conditions (Coote & Hassall, 1973b).

Evidence also exists that histidine, imidazolylpyruvate and imidazolyl-lactate may be inter-converted in *Escherichia coli* B (Hedegaard *et al.*, 1966), *Klebsiella aerogenes*, *Citrobacter freundii*, *Enterobacter cloacae* and *Pseudomonas fluorescens* (Brevet *et al.*, 1968). A histidine aminotransferase activity has also been detected in extracts of *Proteus vulgaris* (Wickramasinghe, 1970). As yet, the enzymology of these reactions in bacteria remains largely obscure. The histidine-transaminating enzyme from *E. coli* B has been partially purified and characterized. It has a broad specificity and is produced by the organism in conditions of low available nitrogen (Wickramasinghe, 1969a,b). Some preliminary findings on the properties of histidine-2-oxoglutarate aminotransferase in cell-free extracts of *Ps. testosteroni* suggested that it too might possess a broad specificity (Coote & Hassall, 1973a). The

present paper records a study of the purification and properties of this enzyme and compares these properties with those of the *E. coli* enzyme, particularly with reference to their respective physiological roles, and with the mammalian histidine aminotransferases, which require pyruvate as the keto acceptor (Spolter & Baldrige, 1964; Emes & Hassall, 1973).

Materials and Methods

Chemicals

Chemicals were purchased from the following sources: cyanogen bromide and *p*-chloroaniline from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; imidazolylpyruvate from Calbiochem Ltd., London W.1, U.K.; L-histidine, 2-oxoglutarate, 4-methyl-2-oxopentanoate, 2-oxobutyrate, *p*-hydroxyphenylpyruvate, phenylpyruvate, pyridoxal 5'-phosphate, 2-mercaptoethanol, glutathione and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride from Sigma Chemical Co., London S.W.6, U.K.; L-histidine hydrochloride from Cambrian Chemicals Ltd., Croydon CR9 3QL, U.K. All other chemicals were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of AnalaR grade where available.

Buffers. Phosphate buffer consisted of KH_2PO_4 with the pH adjusted as required with 5M-NaOH. Tris-acetic acid and Tris-HCl buffers consisted of Tris solutions with the pH adjusted with 5M-acetic acid or 5M-HCl respectively. Buffer concentration is given as the concentration of phosphate or Tris. Borate-arsenate buffer consisted of 0.2M-sodium tetraborate and 0.028M-sodium arsenate with the pH adjusted to 8.0 with 2M-HCl.

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Preparation of affinity-chromatography medium

A two-stage process was used to attach histidine through diaminoethane to a beaded agarose gel (Sephacrose 4B). This length of carbon chain was considered to be sufficient to prevent steric hindrance to protein binding by the gel matrix (Cuatrecasas, 1970).

(1) The Sepharose was activated with 250 mg of CNBr/ml of settled gel at pH 10.0 as described by Cuatrecasas (1970). The activated gel was treated with an equal volume of cold water containing 2 mmol of diaminoethane/ml of settled agarose at pH 10.0. After stirring for 16 h at 4°C, the Sepharose derivative was washed with 2–3 litres of water.

(2) The carboxyl group of L-histidine was coupled to the free amino group of the agarose-bound diaminoethane by the use of a condensing agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide. Histidine (18 mg/ml of settled gel) was added to the washed gel from stage 1 and the pH was adjusted to 4.7 with 2 M-HCl. 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide (25 mg/ml of settled gel) was added over 5 min with continuous stirring and the reaction allowed to proceed at room temperature (15–20°C) for 20 h. The gel was washed with water until the effluent was histidine-free.

Assay of histidine-2-oxoglutarate aminotransferase

Reaction rates were measured in a Gilford 2000 multiple-sample absorbance recorder fitted with a Unicam SP. 500 monochromator. All enzyme assays were conducted at 30°C. Three procedures were used. The most sensitive assay, used as a routine during purification of the enzyme, was based on the method described by Spolter & Baldrige (1963) for the assay of histidine-pyruvate aminotransferase. The formation of the enol-borate complex of imidazolylpyruvate was followed at 293 nm (ϵ 12000) in borate-arsenate buffer (pH 8.0). The complete system (3.5 ml) consisted of sodium tetraborate (500 μ mol), sodium arsenate (70 μ mol), 2-oxoglutarate (100 μ mol), L-histidine (100 μ mol) and enzyme (usually 0.2 ml) preincubated for 10 min with pyridoxal 5'-phosphate (75 μ g/ml). Oxo acid was omitted from the control reaction mixture. When activity towards phenylalanine, tyrosine or tryptophan was assayed, extinction changes were followed at the appropriate wavelength for the enol-borate complex formed (phenylpyruvate, ϵ_{300} 9150; *p*-hydroxyphenylpyruvate, ϵ_{310} 12400; indolylpyruvate, ϵ_{332} 12700).

For studies with the pure enzyme either the utilization or the formation of imidazolylpyruvate was followed spectrophotometrically at 284 nm (ϵ 2250). For measurement of the rate of utilization of imidazolylpyruvate, the reaction mixture (3 ml) contained Tris-acetate buffer, pH 8.0 (300 μ mol), imidazolyl-

pyruvate (1 μ mol) and enzyme preincubated with pyridoxyl 5'-phosphate. The reaction was started by the addition of L-glutamate (20 μ mol) and the E_{284} was measured against a blank mixture that lacked imidazolylpyruvate. When the formation of imidazolylpyruvate was being followed, 2-oxoglutarate (100 μ mol) replaced imidazolylpyruvate in the reaction mixture and the reaction was started by the addition of L-histidine (100 μ mol). The E_{284} was measured against a blank that lacked 2-oxoglutarate.

Units of enzyme activity. One unit of enzyme activity was defined as the utilization or formation of 1 μ mol of imidazolylpyruvate/min at 30°C.

Maintenance and growth of the organism

Pseudomonas testosteroni (N.C.I.B. 10808) was maintained in slope culture on Oxoid nutrient agar. The organism was subcultured monthly and stored at 4°C after incubation for 2 days at 30°C. For purification of histidine-2-oxoglutarate aminotransferase, cells were grown in 100-litre batches on histidine-succinate medium as described by Hassall & Soutar (1974) and harvested in late-exponential phase. They were stored frozen at -18°C until required.

Purification of histidine-2-oxoglutarate aminotransferase

Step 1: preparation of a cell-free extract. The cells were thawed and resuspended in 5 vol. of 0.1 M-potassium phosphate buffer, pH 7.0, at 4°C and then passed three times through a Manton-Gaulin laboratory homogenizer (Hassall & Soutar, 1974) at a pressure of 55 MPa (8000 lbf/in²). After disruption, cell debris was removed by centrifugation at 30000g for 40 min.

Step 2: protamine sulphate treatment. To the supernatant was added protamine sulphate (15 mg/ml in 0.1 M-potassium phosphate buffer, pH 7.0) dropwise, and with continuous stirring, to a final concentration of 1 mg of protamine sulphate/10 mg of protein. The solution was equilibrated for 30 min at room temperature, the resultant precipitate removed by centrifugation (30000g for 20 min) and discarded.

Step 3: ammonium sulphate precipitation. (NH₄)₂SO₄ was added to the supernatant, with stirring, to give 35% of saturation (20.9 g/100 ml) at 4°C. The pH of the solution was maintained at 7.0 by the addition of 2 M-NH₃. After equilibration at 4°C for 30 min the resultant precipitate was removed by centrifugation (38000g for 30 min) and discarded. More (NH₄)₂SO₄ (6.3 g/100 ml) was then added to the supernatant to give 45% of saturation. After equilibration as before, the precipitate was collected by centrifugation and the supernatant

discarded. The precipitate was dissolved in the minimum volume of 0.1M-Tris-HCl buffer-5mM-EDTA (pH8.0) and was dialysed overnight against 0.01M-Tris-HCl buffer-5mM-EDTA (pH8.0).

Step 4: affinity chromatography. The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to a column (20cm $\times$ 3cm) of histidine-treated agarose in 0.01M-Tris-HCl buffer-5mM-EDTA (pH8.0) and was eluted with a linear salt gradient of 0-0.5M-NaCl in the same buffer (total vol. 500ml). Histidine-2-oxoglutarate aminotransferase activity was eluted at a concentration of approx. 0.25M-NaCl.

Step 5: DEAE-cellulose chromatography. Fractions from the affinity-column effluent that contained histidine-2-oxoglutarate aminotransferase activity were pooled and dialysed against 0.01M-potassium phosphate buffer-5mM-EDTA (pH7.0) and were applied to a column (10cm $\times$ 3cm) of DEAE-cellulose which had been equilibrated with this buffer. The column was washed with buffer until the effluent was free of protein, when a linear gradient of 0.01-0.15M-potassium phosphate buffer-15mM-EDTA (pH7.0) in a total volume of 500ml was applied. The enzyme was eluted at a concentration of approx. 0.06M-phosphate.

Step 6: gel filtration. A sample (2ml) of enzyme preparation, concentrated as described below, was subjected to descending gel filtration through a column (110cm $\times$ 5cm) containing Sephadex G-200. The buffer used to pack and elute the column was 0.01M-potassium phosphate-5mM-EDTA, pH7.0. Fractions (2ml) were collected and analysed for histidine-2-oxoglutarate aminotransferase activity and protein. The most active fractions were combined and concentrated.

The purification procedure is summarised in Table 1.

Concentration of protein samples

Protein solution was applied to a small column (4.0cm $\times$ 0.5cm) of DEAE-cellulose, equilibrated with 0.01M-potassium phosphate buffer-5mM-EDTA (pH7.0), and was recovered by frontal elution with 0.1M-buffer.

Determination of protein

Protein was measured by the Folin-Ciocalteu method as described by Lowry *et al.* (1951), with freeze-dried bovine serum albumin as standard.

Electrophoresis of protein

Polyacrylamide-disc-gel electrophoresis was performed at pH8.6 as described by Davis (1964). Gels at a concentration of 7.5% (w/v) acrylamide were used. Amido Black [0.1% in 7% (v/v) acetic acid] was used to stain proteins.

Assay of histidine-2-oxoglutarate aminotransferase activity on polyacrylamide gels

Histidine-2-oxoglutarate aminotransferase was located on polyacrylamide gels by using the method described by Emes & Hassall (1973) for histidine-pyruvate aminotransferase but with 2-oxoglutarate (25mM) replacing pyruvate. Aminotransferase bands are revealed by the formation of the enol-borate complex of imidazolylpyruvate in the gel.

Chromatography of samples on Sephadex G-200

For an approximate determination of the molecular weight of histidine-2-oxoglutarate aminotransferase, the enzyme was chromatographed on Sephadex G-200 as described by Andrews (1965). A column (110cm $\times$ 5cm) of gel was equilibrated in 0.1M-Tris-HCl buffer (pH8.5) and calibrated by measuring the elution volumes of substances of known molecular weights (^{131}I -labelled insulin B chain, 3500; cytochrome *c*, 12400; bovine serum albumin, 68000; alkaline phosphatase, 75000; lactate dehydrogenase, 130000; histidase, 200000; β -galactosidase, 510000; Blue Dextran, approx. 2×10^6). The sample volume was 2ml for each chromatography run.

Identification of reaction products by paper chromatography

Reaction mixtures (3.0ml) were prepared containing, in 0.1M-Tris-acetate buffer, pH8.0, (a) L-histidine (300 μmol)+2-oxoglutarate (300 μmol) and

Table 1. Purification of histidine-2-oxoglutarate aminotransferase

One unit of activity is the amount of enzyme catalysing the formation of 1 μmol of imidazolylpyruvate enol-borate complex/min at 30°C.

Procedure	Vol. (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)
Crude extract	1430	1.09	10.4	0.105	100
35-45%-satd. $(\text{NH}_4)_2\text{SO}_4$	105	9.95	43.3	0.230	67
Affinity chromatography	94	7.30	1.42	5.14	44
DEAE-cellulose chromatography	85	3.70	0.22	16.8	20
Sephadex G-200 chromatography	70	1.78	0.10	17.8	8

(b) imidazolylpyruvate (300 μ mol) + L-glutamate (300 μ mol). After incubation with enzyme at 30°C for 1 h the reactions were terminated by heating to 100°C and then adding an equal volume of 1 M-HCl. Protein was removed by centrifugation and the supernatant solutions were applied to paper chromatograms. Two chromatographic systems were used. (i) Amino acids were separated by ascending chromatography on Whatman no. 1 paper in butan-1-ol-pyridine-water (1:1:1, by vol.). The chromatograms were left to dry in a current of air at room temperature and were dipped in 0.2% (w/v) ninhydrin in acetone. Spots were developed by heating at 50°C for a few minutes. (ii) Oxo acids and imidazolyl compounds were separated by ascending chromatography overnight on Whatman no. 1 paper in the organic phase of 2-methylpropan-1-ol-formic acid-water (19:2:6, by vol.) as solvent (Hassall & Greenberg, 1963). After drying the paper in air at room temperature, 2-oxoglutarate spots were detected by their absorption of u.v. light. Imidazolyl compounds were detected by spraying chromatograms with 3% (w/v) *p*-chloroaniline in 10% (v/v) HCl-aq. 5% (w/v) NaNO₃ (1:1, by vol.). Red spots developed on a yellow background when the chromatogram was placed in an atmosphere of NH₃ (Hall, 1952).

By comparison of R_F values with those of marker solutions, the reaction scheme:

was confirmed.

Determination of Michaelis constants

Kinetic measurements were carried out in Tris-acetate buffer at pH 8.0. Concentration-independent Michaelis constants were obtained for imidazolylpyruvate and L-glutamate by the method of Velick & Vavra (1962). Apparent Michaelis constants were determined for L-histidine and 2-oxoglutarate as described by Lineweaver & Burk (1934), with the second substrate at a saturating concentration. Reaction rates were measured at a minimum of six different substrate concentrations in each case and plots of rate^{-1} against substrate concn.^{-1} were linear, the straight lines being fitted to the points visually. The apparent Michaelis constant for pyridoxal 5'-phosphate was determined similarly.

Determination of equilibrium constant

Reaction mixtures (3.0 ml) were prepared containing the four substrates at various concentrations in the range 0.5–2.0 mM, pyridoxal 5'-phosphate (100 μ g), Tris-acetate buffer, pH 8.0 (300 μ mol), EDTA (15 μ mol) and enzyme. Reactions were started by

the addition of either L-glutamate or L-histidine. The E_{284} of each mixture was measured before starting the reaction and at intervals afterwards until it became constant. The concentration of imidazolylpyruvate was determined from the final extinction value. The concentrations of the other reactants were calculated from this and their initial concentrations.

Results

Purity of preparations

Disc electrophoresis of samples of the peak fractions from Sephadex G-200 gel filtration revealed a single protein band the mobility of which corresponded to the u.v.-absorbing band detected by the gel assay for enzyme activity. Concentration of these fractions on DEAE-cellulose gave a preparation having only 20% of the activity of that applied to the column and giving a number of protein-staining bands when subjected to polyacrylamide-gel electrophoresis. The single-banded material was used in all the studies reported.

Stability

Purified preparations of histidine-2-oxoglutarate aminotransferase were unstable; activity losses during purification procedures and storage were kept to a minimum by the inclusion of reagents which prevented metal-catalysed oxidations of labile thiol groups (Fig. 1). Similar findings were recorded for alanine aminotransferase (Saier & Jenkins,

Fig. 1. Stabilization of histidine-2-oxoglutarate aminotransferase activity by EDTA and reduced thiol compounds

Purified histidine-2-oxoglutarate aminotransferase was stored at approx. 0.1 mg/ml in 0.1 M-Tris-acetate buffer, pH 8.0, at 4°C and in the presence of the following: ○, no further additions; □, 10 mM-L-cysteine; ▲, 10 mM-GSH; ■, 10 mM-mercaptoethanol; △, 10 mM-dithiothreitol; ●, 5 mM-EDTA. Samples were withdrawn at the times shown and preincubated with pyridoxal 5'-phosphate before assay.

Fig. 2. Effect of varying the concentration of imidazolylpyruvate on the double-reciprocal plot of reaction rate against concentrations of L-glutamate for histidine-2-oxoglutarate aminotransferase

The reactions were carried out at pH 8.0 in 0.1M-Tris-acetate buffer at the following concentrations of imidazolylpyruvate: Δ , 0.4 mM; $\blacksquare$, 0.5 mM; $\blacktriangle$, 0.6 mM; $\square$, 0.7 mM.

Fig. 3. Effect of varying the concentration of L-glutamate on the double-reciprocal plot of reaction rate against concentration of imidazolylpyruvate for histidine-2-oxoglutarate aminotransferase

The reactions were carried out at pH 8.0 in 0.1M-Tris-acetate buffer at the following concentrations of L-glutamate: Δ , 0.25 mM; $\blacksquare$, 0.3 mM; $\blacktriangle$, 0.4 mM; $\square$, 0.5 mM; $\bullet$, 1.0 mM.

1967). Thus EDTA (5 mM) was included in buffer solutions used during purification procedures and storage. Dithiothreitol and 2-mercaptoethanol were found to have a similar stabilizing effect, but did not improve on the results obtained by using EDTA.

In the course of the purification procedure a total loss of aminotransferase activity occurred which could be restored by the addition of pyridoxal 5'-phosphate. The resolution of apoenzyme and coenzyme was particularly high during affinity and ion-exchange chromatography, but increases in activity of up to 40% were obtained by addition of pyridoxal 5'-phosphate to crude cell-free extracts. This resolution of cofactor may also have been a factor in the irreversible loss of activity, since the holoenzyme forms of pyridoxal 5'-phosphate-dependent enzymes are frequently more resistant to denaturation than are the corresponding apoenzymes (Holten *et al.*, 1967; Morino & Snell, 1967; Tate & Meister, 1968).

Molecular weight

The enzyme was eluted from Sephadex G-200 between bovine serum albumin and alkaline phosphatase, where the ratio of elution volume to exclusion volume was 2.0–2.1. This corresponds to a molecular weight of approximately 70000.

Reaction mechanism and Michaelis constants

The reaction was measured by following the disappearance of imidazolylpyruvate in the presence

of glutamate. Double-reciprocal plots of initial velocity against concentration of one substrate at a series of fixed concentrations of the second substrate gave a series of parallel straight lines (Figs. 2 and 3). The reaction mechanism is thus one involving only binary substrate complexes and may be termed Ping Pong (Cleland, 1963). Apparent Michaelis constants and apparent maximum velocities at each concentration of the second substrate were determined from the intercepts of these plots. Double-reciprocal plots of apparent Michaelis constants and apparent maximum velocities so obtained, against the concentration of the second substrate, again gave straight lines from which concentration-independent Michaelis constants were determined for imidazolylpyruvate (2.5 mM) and L-glutamate (5 mM).

Apparent Michaelis constants for L-histidine (4 mM) and 2-oxoglutarate (5.3 mM) were determined where the concentration of the second substrate was kinetically saturating (33 mM in each case). The apparent Michaelis constant for pyridoxal 5'-phosphate (59 mM) was also determined in the presence of 33 mM-L-histidine and 33 mM-2-oxoglutarate.

Equilibrium constant

The equilibrium constant

$$\frac{[\text{L-histidine}][2\text{-oxoglutarate}]}{[\text{imidazolylpyruvate}][\text{L-glutamate}]}$$

was 0.49 at 30°C in Tris-acetate buffer, pH 8.0.

Table 2. *Substrate specificity of purified histidine-2-oxoglutarate aminotransferase*

Activity was measured by the enol-borate assay system with the minor modification that where amino acid specificity was examined the reaction mixture contained 20 μ mol of the amino acid. In all other cases, 100 μ mol of each substrate was present. The values in parentheses show the relative rates previously obtained by using crude extracts containing the enzyme (Coote & Hassall, 1973a).

Substrates present in the reaction mixture	Reaction rate (nmol/min)	Relative reaction rate
2-Oxoglutarate		
+L-Histidine	52.0	100 (100)
+L-Tyrosine	3.6	7 (83)
+DL-Phenylalanine	5.2	10 (58)
+L-Tryptophan	3.1	6 (—)
+D-Histidine	0	0 (0)
+5'-N-Methyl-L-histidine	0	0 (0)
L-Histidine		
+2-Oxoglutarate	60.0	100 (100)
+2-Oxobutyrate	1.8	3 (8)
+4-Methyl-2-oxopentanoate	1.8	3 (6)
+2-Oxomalonnate	1.2	2 (—)
+Pyruvate	0	0 (6)

pH optimum

The activity of histidine-2-oxoglutarate aminotransferase was investigated as a function of pH over the range pH5.0–9.5. Enzyme assays were performed by using imidazoly pyruvate and L-glutamate as substrates. The molar extinction coefficient of imidazoly pyruvate was determined at each pH used. Phosphate buffer (cuvette concentration 0.1 M) was used between pH5.0 and 7.8 and Tris-acetate (0.1 M) between pH7.0 and 9.5. Differences in aminotransferase activities in the overlap range owing to the buffers were considered to be negligible. The enzyme possessed optimal activity in the range pH7.5–8.5, the maximum being recorded at pH8.0. Outside this range activity decreased sharply.

Specificity

Aminotransferase activity towards aromatic amino acids and analogues of 2-oxoglutarate was measured by using the borate assay system. The rates relative to the normal substrates are given in Table 2.

Histidine-2-oxoglutarate aminotransferase was also found to have no activity towards L-histidinol phosphate as measured by the assay system of Martin & Goldberger (1967) for imidazolyacetol phosphate-L-glutamate aminotransferase (EC 2.6.1.9).

Table 3. *Inhibition of histidine-2-oxoglutarate aminotransferase by p-chloromercuribenzoate*

Enzyme activity was measured in 0.1 M-Tris-acetate buffer by following the formation or utilization of imidazoly pyruvate. The enzyme was preincubated with p-chloromercuribenzoate for 15 min before beginning the reaction. Rates are expressed as a percentage of the uninhibited rates (40 nmol of imidazoly pyruvate formed/min in the forward direction; 19 nmol utilized/min when assayed in the reverse direction).

Assay conditions	Activity remaining (%)
0.05 mM-p-Chloromercuribenzoate	9
0.1 mM-p-Chloromercuribenzoate	0
0.1 mM-p-Chloromercuribenzoate after the addition of assay concentrations of	
2-Oxoglutarate (33 mM)	87
Imidazoly pyruvate (0.33 mM)	82
L-Histidine (33 mM)	66
L-Glutamate (6.6 mM)	59
0.1 mM-p-Chloromercuribenzoate and subsequent addition of	
1.0 mM-L-Cysteine	81
1.0 mM-2-Mercaptoethanol	73

Inhibition

Complete inhibition of histidine-2-oxoglutarate aminotransferase was obtained at a final cuvette concentration of 0.1 mM-p-chloromercuribenzoate. Some 80% of the original activity could be restored by addition of 1 mM-cysteine or 1 mM-2-mercaptoethanol. Addition of oxo rather than amino substrates before the inhibitor proved to be a more effective method of preventing reactive thiol groups combining with p-chloromercuribenzoate (Table 3).

In an experiment to investigate inhibition by NaBH₄, a sample of enzyme, fully resolved from pyridoxal phosphate, was incubated for 30 min with 0.5 mM-NaBH₄ in 0.1 M-Tris-acetate buffer, pH8.0, and desalted by chromatography on Sephadex G-25. An identical sample was treated similarly after the addition of 75 μ g pyridoxal 5'-phosphate/ml. A control sample of enzyme was passed through Sephadex G-25 without NaBH₄ treatment. Pyridoxal 5'-phosphate was added to all three samples of enzyme before assay. The control sample retained 78% of its initial activity. The sample incubated with NaBH₄ retained 65% of its activity, but only 4% of the initial activity was recovered from the sample incubated with NaBH₄ and pyridoxal 5'-phosphate. These results suggest that NaBH₄ inhibition was caused by reduction of an aldimine bond between pyridoxal phosphate and the apoenzyme.

Discussion

Metal-ion-catalysed oxidation of thiol groups was considered to be a contributory factor to inactivation of the enzyme during purification and storage, since some protection was achieved by the inclusion of EDTA in buffer solutions. The involvement of thiol groups in the aminotransferase reaction was further indicated by *p*-chloromercuribenzoate inhibition. Some measure of protection against this inhibition was obtained by incubation of the enzyme with oxo acid substrates before the addition of the inhibitor. Similar findings were recorded for aspartate aminotransferase (Karni-Katsadimas *et al.*, 1969) and were attributed to the conformation of the oxo acid-enzyme complex rendering the thiol groups less susceptible to attack. In all probability such effects also explain the reported stabilization of other aminotransferase enzymes by keto substrates (Jenkins *et al.*, 1959; Scardi *et al.*, 1963). However, the inclusion of oxo acid substrates in buffers proved of little value in preventing activity losses during this work.

The ready resolution of pyridoxal 5'-phosphate and apoenzyme enabled the measurement of a Michaelis constant of 5.9×10^{-8} M for pyridoxal 5'-phosphate. A similar value (1.67×10^{-8} M) was recorded for rat liver tyrosine aminotransferase, which could be resolved with corresponding ease (Hayashi *et al.*, 1967). Despite this ease of resolution, the characteristics of NaBH_4 inhibition suggest the existence of an aldimine bond between pyridoxal 5'-phosphate and the apoenzyme.

Preliminary studies on histidine-2-oxoglutarate aminotransferase in cell-free extracts of *Ps. testosteroni* indicated a broad specificity to aromatic amino acids (Coote & Hassall, 1973a), but the purified enzyme retained only slight activity towards phenylalanine, tyrosine and tryptophan, suggesting that other aminotransferase activities were being assayed in the unfractionated system. Within the limits of the oxo acids tested the enzyme was specific to 2-oxoglutarate. This enzyme thus exhibits a greater specificity than either the rat liver histidine-pyruvate aminotransferases (Emes & Hassall, 1973) or the histidine-transaminating enzyme of *E. coli* B (Wickramasinghe, 1969a). The latter enzyme possesses activity towards histidine, phenylalanine, tyrosine, tryptophan and lysine, with pyruvate functioning as an amino acceptor in addition to 2-oxoglutarate. It seems unlikely therefore that the *Ps. testosteroni* enzyme could fulfil the role postulated for the *E. coli* B enzyme, namely that it is produced under conditions of nitrogen starvation and has a broad specificity in order to utilize as many amino acids as possible. Further, no evidence was obtained in the present work for inhibition of histidine-2-oxoglutarate aminotransferase by imidazolyl-lactate

sufficient to prevent a build-up of this compound before its excretion by the cell, as was proposed for *E. coli* B (Wickramasinghe, 1969b).

No aminotransferase activity could be detected between 2-oxoglutarate and L-histidinol phosphate, the reaction catalysed by imidazolylacetol phosphate-L-glutamate aminotransferase, which is the eighth step in histidine biosynthesis. Thus in this respect *Ps. testosteroni* does not resemble *Salmonella typhimurium*, where extracts were found to possess a histidine-2-oxoglutarate aminotransferase activity which could be attributed to the biosynthetic enzyme (Albritton & Levin, 1969).

The apparent Michaelis constant for histidine determined in the present work (4.0×10^{-3} M) is of a similar order to those reported for rat liver histidine-pyruvate aminotransferases (Spolter & Baldrige, 1964; Emes & Hassall, 1973) but contrasts sharply with the unusually high value obtained for the *E. coli* B enzyme (1.37×10^{-1} M) (Wickramasinghe, 1969a). This finding appears rather paradoxical in the light of the directions in which these enzymes are thought to operate physiologically. The Michaelis constant for imidazolylpyruvate was, however, lower than that for histidine with the *Ps. testosteroni* enzyme, although when these values are compared it must be remembered that the histidine value was an apparent term and that for imidazolylpyruvate was a concentration-independent term.

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References

- Albritton, W. L. & Levin, A. P. (1969) *Biochem. J.* **114**, 662-664
- Andrews, P. (1965) *Biochem. J.* **96**, 595-606
- Brevet, A., Hoffmeyer, J., Roche, J. & Hedegaard, J. (1968) *C. R. Soc. Biol.* **162**, 1054-1058
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104-137
- Coote, J. G. & Hassall, H. (1973a) *Biochem. J.* **132**, 409-422
- Coote, J. G. & Hassall, H. (1973b) *Biochem. J.* **132**, 423-433
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059-3065
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404-427
- Emes, A. V. & Hassall, H. (1973) *Biochem. J.* **136**, 649-658
- Hall, D. A. (1952) *Biochem. J.* **51**, 499-504
- Hassall, H. (1966) *Biochem. J.* **101**, 22P
- Hassall, H. & Greenberg, D. M. (1963) *J. Biol. Chem.* **238**, 1423-1431
- Hassall, H. & Soutar, A. K. (1974) *Biochem. J.* **137**, 559-566
- Hayashi, S., Granner, D. K. & Tomkins, G. M. (1967) *J. Biol. Chem.* **242**, 3998-4006
- Hedegaard, J., Brevet, J. & Roche, J. (1966) *Biochem. Biophys. Res. Commun.* **25**, 335-339

- Holten, D., Wicks, W. D. & Kenney, F. T. (1967) *J. Biol. Chem.* **242**, 1053–1059
- Jenkins, W. T., Yphantis, D. A. & Sizer, I. W. (1959) *J. Biol. Chem.* **234**, 51–57
- Karni-Katsadimas, I., Dimitropoulos, C. & Evangelopoulos, A. E. (1969) *Eur. J. Biochem.* **8**, 50–54
- Lineweaver, H. & Burk, D. (1934) *J. Amer. Chem. Soc.* **56**, 658–666
- Lowry, O. H., Rosebrough, W. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, R. G. & Goldberger, R. F. (1967) *J. Biol. Chem.* **242**, 1168–1174
- Morino, Y. & Snell, E. E. (1967) *J. Biol. Chem.* **242**, 5591–5601
- Saier, M. H. & Jenkins, W. T. (1967) *J. Biol. Chem.* **242**, 91–100
- Scardi, V., Scotto, P., Iaccarino, M. & Scarano, E. (1963) *Biochem. J.* **88**, 172–175
- Spolter, P. D. & Baldrige, R. C. (1963) *J. Biol. Chem.* **238**, 2071–2074
- Spolter, P. D. & Baldrige, R. C. (1964) *Biochim. Biophys. Acta* **90**, 287–290
- Tate, S. S. & Meister, A. (1968) *Biochemistry* **7**, 3240–3247
- Velick, S. F. & Vavra, J. (1962) *J. Biol. Chem.* **237**, 2109–2122
- Wickramasinghe, R. H. (1969a) *Enzymologia* **36**, 161–171
- Wickramasinghe, R. H. (1969b) *Enzymologia* **37**, 91–96
- Wickramasinghe, R. H. (1970) *Experientia* **26**, 37–38