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PREPARATION AND PROPERTIES OF ORNITHINE-OXO-ACID AMINOTRANSFERASE OF RAT KIDNEY

COMPARISON WITH THE LIVER ENZYME

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Summary

Ornithine-oxo-acid aminotransferase (EC 2.6.1.13) from rat kidney was prepared as a single homogeneous protein as judged by polyacrylamide gel electrophoresis, ultracentrifuge analysis and double diffusion precipitin test. Content of pyridoxal phosphate, light absorption spectra, circular dicroism spectra, K_m values, inhibitors, and electrophoretic mobilities of the proteins after reactions with group modifying reagents were similar for the ornithine-oxo-acid aminotransferases of rat kidney and liver. Rates of reaction with group modifying reagents, stabilities to storage at —15°C, and stabilities to temperatures above 55°C differed significantly for the two enzymes. The liver enzyme contained two more cysteine residues than the kidney enzyme as determined by three different methods. Heating the liver enzyme at 66—67°C at pH 5.9 for 1 h decreased the thiol groups titratable by 5,5′-dithio-bis(2-nitrobenzoic acid)(Nbs_2). Under the same conditions titratable thiol groups of the kidney enzyme were not decreased. Amino acid analysis revealed probably significant differences in tyrosine and isoleucine content in addition to cysteine. It was concluded that the primary structures of ornithine-oxo-acid aminotransferases of rat liver and kidney are not fully identical.

Introduction

Ornithine-oxo-acid aminotransferase (L-ornithine-2-oxo-acid aminotransferase; EC 2.6.1.13) catalyzes a reversible transamination between ornithine and α-ketoglutarate, this reaction being at the nexus of the metabolic sequences in-

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Abbreviations: Nbs_2, 5,5′-dithio-bis(2-nitrobenzoic acid); $ClHgBzO^-$, *p*-chloromercuribenzoate.

terconverting glutamate, proline and arginine [1]. This enzyme is especially active in rat liver and kidney. In many instances the changes of enzyme activity in the two organs were not parallel or were even in opposite directions as in the case of high concentrations of glutamate or arginine in diets and various other dietary input or hormonal administration [2—11]. If these functional differences did indeed obtain they could conceivably be reflected in structural differences in the enzymes from these two tissues. With regard to this point, two groups of investigators have reported some comparative data obtained with purified ornithine-oxo-acid aminotransferase from liver and kidney and concluded that the two proteins are identical [12,13]. To elucidate further the molecular mechanism underlying the differential regulation of ornithine-oxo-acid aminotransferase from liver and kidney, we have investigated in detail the structures of these and observed many similarities and differences which are reported here.

Methods

Purification and crystallization of ornithine-oxo-acid aminotransferase from rat kidney and liver. The purification procedure was derived from the previous descriptions of Strecker [1], and Peraino et al. [14] for the liver enzyme and Katanuma and colleagues [12,15,16] for the liver and kidney enzymes. Briefly, the method consists of obtaining mitochondria from rat liver or kidney, solubilizing the enzyme by ultrasonication, $(NH_4)_2SO_4$ fractionation, trituration with pyridoxal phosphate followed by dialysis, acid heat step followed by second $(NH_4)_2SO_4$ fractionation and finally by crystallization. The specific activities for the final purified crystalline preparations varied from 1200 to 1500 units per mg protein with an average of 1350.

Enzyme assay. This was done spectrophotometrically as described earlier [1,12,15,16]. One unit is defined as 1 μmol of Δ^1-pyrroline-5-carboxylate produced per h.

Polyacrylamide disc gel electrophoresis. Polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue was conducted as described by Maizel [18]. Resolving gels of 10% acrylamide were used; 8 M urea or 0.1% sodium dodecyl sulfate was incorporated in gels and buffers, as required. Enzyme preparations analyzed were either untreated, mixed with 8 M urea, or heated for 2 min with sodium dodecyl sulfate and 2-mercaptoethanol at 95°C [18].

Ultracentrifugation analysis. Sedimentation velocity experiments were conducted with a Beckman-Spinco Model E analytical ultracentrifuge equipped with Schlieren optics and using the A_n-D rotor with single-sector cells. Photographs were taken at a running speed of 59 780 rev./min at 20°C. Boundary positions were measured with a Nikon Optical Microcomparator.

Sedimentation equilibrium analysis was carried out according to Yphantis [19] using the Beckman-Spinco Model E ultracentrifuge equipped with Rayleigh interference optics and an RTIC temperature control unit. The protein solutions were centrifuged in the A_n-D rotor at 20°C using a standard double sector cell. Given a column height of 0.6 mm, the experimental conditions were sufficient to essentially deplete the meniscus of protein. Fringe patterns were

photographed and measurements of fringe displacement against distance from the axis of rotation were made with a Nikon comparator. Partial specific volume of kidney ornithine-oxo-acid aminotransferase was calculated from its amino acid composition.

Modification reactions. Maleylation was conducted according to Butler et al. [20] except that maleic anhydride was present at a 30-fold excess over the lysyl residues in the enzyme, and the reaction mixture, after the last addition of maleic anhydride, was incubated for 2 h in an ice bath. Succinylation was performed by the method of Klotz and Keresztes-Nagy [21] except that succinic anhydride was present at 30-fold excess over the lysyl residues in the enzyme and the reaction mixture was incubated overnight at room temperature. Reaction with *N*-ethylmaleimide was carried out as described by Colman and Chu [22] except that the *N*-ethylmaleimide was present at 1000-fold excess over protein and the incubation was continued for 1 h at 30°C. Carboxymethylation with iodoacetate was conducted by the procedure of Edelstein et al. [23] for reaction with iodoacetamide. Acetylation with acetic anhydride was performed according to Frankel-Conrat [24].

Preparation and use of antisera. Enzyme dissolved in potassium phosphate/pyridoxal phosphate solution (0.01 M potassium phosphate, pH 6.8, containing 25 μg/ml of pyridoxal phosphate) was emulsified together with an equal volume of complete Freunds adjuvant. A total of 1 mg of emulsified protein was injected into each of the four footpads of New Zealand white rabbits. After 4 weeks, booster inoculations of 1 mg of fresh emulsified enzyme protein was injected intramuscularly into each leg of the rabbits. After an additional 2 weeks the rabbits were bled from the central ear artery, the collected blood clotted, and the serum collected. Agar plates for the double diffusion precipitin test were prepared and used as described by Ouchterlony [25].

Pyridoxal phosphate bound to enzyme was assayed fluorimetrically by the method of Adams [26], using pyridoxal phosphate as a standard. Pyridoxal was assayed fluorimetrically by the method of Toepfer et al. [27] using pyridoxal as standard. B_6 activity was determined by microbiological assay using *Saccharomyces carlsbergensis* as described by Haskell and Snell [28] with pyridoxal phosphate as a stantard.

Circular dichroism spectra. Circular dichroism spectra were obtained with the Cary Model 60 Recording Spectropolarimeter with a 6001 CD attachment. Measurements were made in the visible and near ultraviolet ranges at a temperature of 27°C in a cell with a light path of 1 cm. Molar ellipticities, $[\theta]$, were calculated from the following equation, $[\theta] = \theta \cdot \text{MRW}/10 \cdot l \cdot c$, where θ is the observed ellipticity at wavelength λ, MRW is the mean residue weight which was taken to be 110, l is the length of the light path in cm and c is the protein concentration in g per ml. The slit widths of the polarimeter were programmed to maintain a wavelength resolution of better than ±0.75 nm in the entire spectral range studied. Absorptions were also less than 2.0, protein concentrations being adjusted accordingly. Calculations of secondary forms of structure were performed by a computer analysis program as described by Listowsky et al. [29].

Amino acid analysis. The aliquots of enzyme in 6 M HCl were hydrolyzed and analyzed in the TSM (Technicon Instrument Co., Tarrytown, N.Y.) amino

acid analyzer. To determine cysteine content, other samples of enzyme were oxidized to cysteic acid with performic acid according to Hirs [30] or to Moore [31]. Alternatively, to convert cysteine to carboxymethyl cysteine residues, samples of enzyme were treated with iodoacetate as described by Edelstein et al. [23]; before being analyzed, known concentrations of carboxymethyl cysteine were used as standards. Tryptophan was determined according to Spande and Witkop [32]. Titration of protein with 5,5'-dithio-bis(2-nitrobenzoic acid) (Nbs_2) was conducted according to Ellman [33], in a N_2 atmosphere, either with or without addition of sodium dodecyl sulfate to a final concentration of 2% as described in the text or legends. For calculating thiol group content an extinction coefficient of 13 600 $\cdot$ M^{-1} $\cdot$ cm^{-1} at 410 nm for the *p*-nitrophenol anion was used [33].

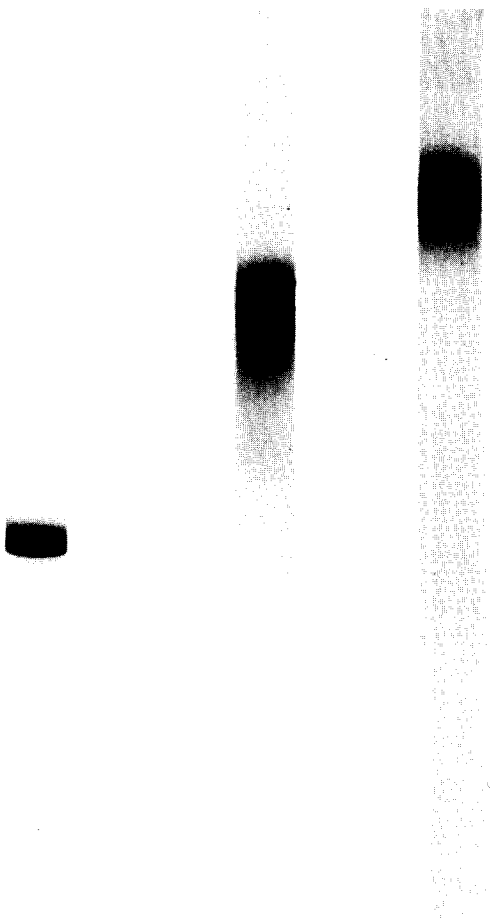

Fig. 1. Electrophoresis of purified kidney ornithine-oxo-acid aminotransferase in, from left to right, sodium dodecyl sulfate-polyacrylamide gel, polyacrylamide-urea gel and polyacrylamide gel. The gels were prepared as described under Methods, and stained with Coomassie brilliant blue. Each gel contains approx. 10 μg of kidney enzyme.

Results

Criteria of purity. The homogeneity of purified enzyme preparations was determined by polyacrylamide gel electrophoresis, sedimentation and high specific activity.

Fig. 1 compares stained gels after electrophoresis of samples of purified rat kidney ornithine-oxo-acid aminotransferase subjected to different treatments. Three types of gel preparations were used, polyacrylamide, urea-polyacrylamide and sodium dodecyl sulfate-polyacrylamide. In each instance the purified enzyme migrated as a single band. In the absence of urea or sodium dodoecyl sulfate, the band was somewhat more diffuse perhaps due to aggregation. As described below, the band obtained in sodium dodecyl sulfate-polyacrylamide corresponded to a subunit of one-fourth the molecular weight of the holoenzyme.

Like other workers [12], we also observed a single peak on ultracentrifugal analysis. Sedimentation velocities for the kidney enzyme were determined at protein concentrations ranging from 2 to 8 mg/ml. Symmetrical patterns were obtained; one of these is reproduced in Fig. 2. $S_{20,w}$ was calculated to be 10.45.

Immunological homogeneity of the purified kidney enzyme was tested by the Ouchterlony double-diffusion technique [25] and showed a single precipitation line. No precipitation band was obtained with antiserum to bovine serum albumin. Fig. 3 is a photograph of an Ouchterlony plate with kidney and

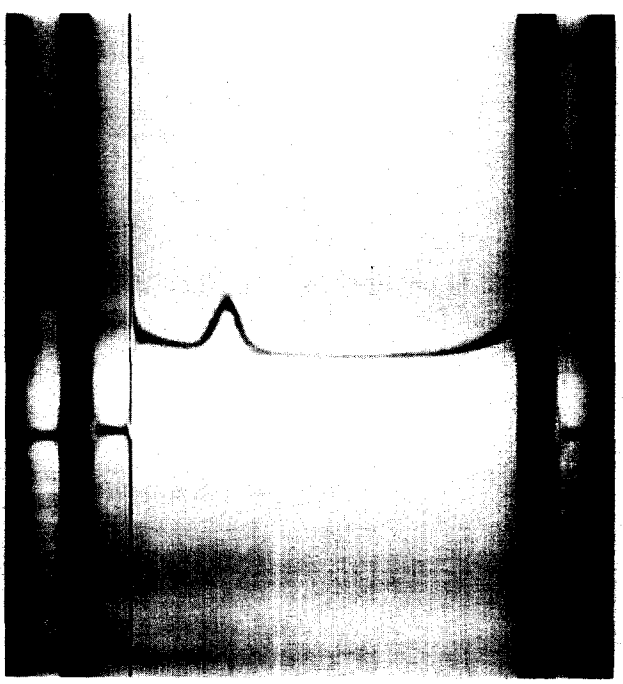

Fig. 2. Sedimentation velocity of purified rat kidney ornithine-oxo-acid aminotransferase at a protein concentration of 2.33 mg per ml in 0.01 M potassium phosphate buffer, pH 6.8. The experiment was conducted at 20°C at 56 890 rev./min and a bar angle at 65°. Direction of sedimentation is from left to right.

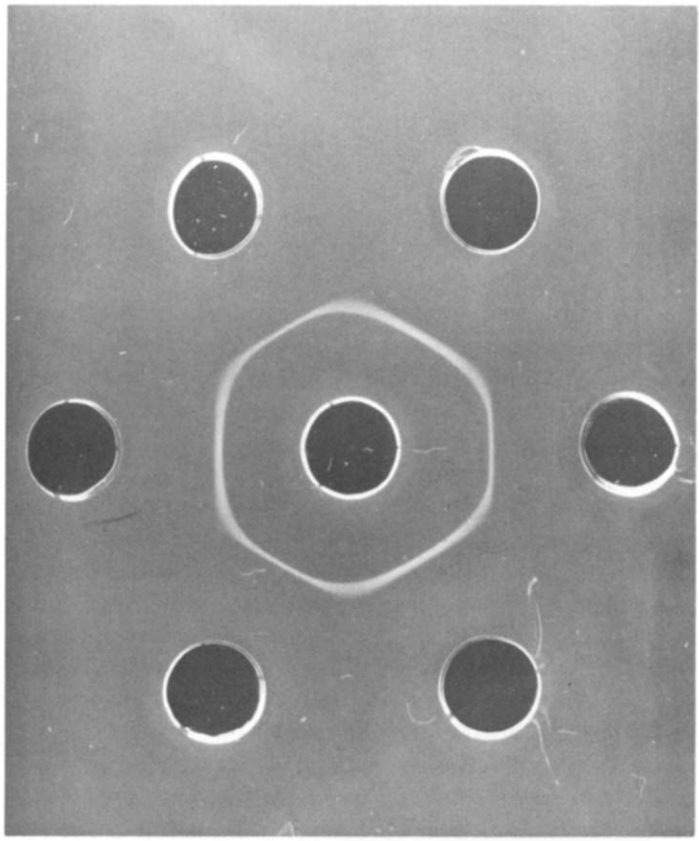

Fig. 3. Double-diffusion pattern of liver and kidney ornithine-oxo-acid aminotransferase. Center well contained rat kidney ornithine-oxo-acid aminotransferase antibody. Outer wells contained kidney and liver enzyme antigens, alternately placed. All wells contained approx. 5—10 μg of enzyme.

liver enzymes diffused against antisera to the kidney enzyme. It can be seen that single precipitation lines are formed and that the lines for the liver and kidney enzymes fuse without spur formation. Identical patterns were obtained when the liver and kidney enzymes were diffused against antisera to the liver enzyme.

Stability. Previous work had suggested that exogenous pyridoxal phosphate was required for maintenance of enzymic activity [1]. Although this indeed may be true for less purified preparations, or for enzyme in solutions of alkaline pH, preparations of ornithine-oxo-acid aminotransferase after the second $(NH_4)_2SO_4$ step were equally stable in 0.01 M potassium phosphate, pH 6.8, as in the same buffer with pyridoxal phosphate added. Homogeneous preparations of enzyme, from either liver or kidney, lost activity slowly during storage at −15°C; the liver enzyme showed somewhat greater losses in this regard. Storage at pH 6.8 appeared to be most effective for preservation of activity of preparations of the liver enzyme. The kidney enzyme was equally stable at pH 8.0 as at 6.8. At pH 6.8, in 0.01 M potassium phosphate buffer at −15°C, about 70% of the liver enzyme activity and 80% of the kidney enzyme activity remained after 4 months.

Determinations of enzyme activity always resulted in higher values when the stock solution of enzyme was diluted in a solution containing 2 mg per ml of bovine serum albumin. With cruder fractions dilution in bovine serum albumin did not affect the activity.

Molecular weight. The molecular weight of rat kidney ornithine-oxo-acid aminotransferase was determined by equilibrium sedimentation according to Yphantis [19]. The molecular weights of the subunits of these enzymes were obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by comparison of electrophoretic mobility with that of proteins of known molecular weights [35—37]. For equilibrium sedimentation analysis a sample of the kidney enzyme was centrifuged in the analytical rotor, A_n-D, at 12 590 rev./min, for 24 h at 20°C after the centrifuge had reached the desired velocity. Fig. 4 presents a plot of the natural logarithm of the concentration of protein versus the distance from the axis of rotation in cm^2. The slope of the straight line so obtained corresponds to a molecular weight of 161 000 and up to the maximum concentration in the distribution there was no definitive evidence of aggregation or other polydispersity. The relative electrophoretic mobilities of the sodium dodecyl sulfate-dissociated protein chains of the liver and kidney enzymes were the same, and were estimated to have a molecular weight of about

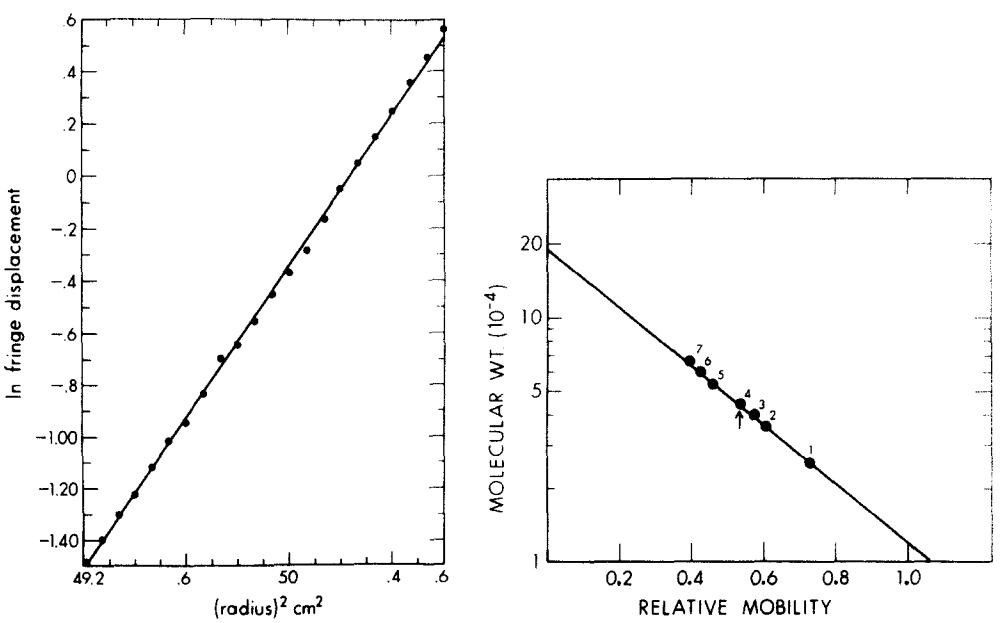

Fig. 4. High speed sedimentation equilibrium data of purified rat kidney ornithine-oxo-acid aminotransferase. The protein concentration was 0.8 mg per ml in 0.01 M potassium phosphate buffer, pH 6.8. Sedimentation was at 12 590 rev./min for 24 h at 20°C using a multichannel double sector cell.

Fig. 5. Electrophoresis on sodium dodecyl sulfate-containing polyacrylamide gels. The molecular weight, on logarithmic scale, is plotted with respect to mobility on the gels. The marker proteins of known molecular weight were: 1, chymotrypsinogen, 25 000; 2, lactate dehydrogenase, 36 000; 3, aldolase, 40 000; 4, ovalbumin, 43 500; 5, glutamate dehydrogenase, 53 000; 6, catalese, 60 000 and 7, bovine serum albumin, 66 000. The mobility of liver and kidney ornithine-oxo-acid aminotransferase is indicated by the arrow which corresponds to a molecular weight of 43 000 ± 500.

43 000 (Fig. 5). The protein chains of either enzyme comigrated with ovalbumin which has been reported to have a molecular weight of 43 500. This value, approximately one-fourth of the molecular weight of the homoenzyme (161 000), suggests that both enzymes are composed of four subunits.

Pyridoxal phosphate. Both liver and kidney ornithine-oxo-acid aminotransferase have been reported to contain two molecules of pyridoxal phosphate per molecule of enzyme [12,16]. The corresponding determinations in our laboratory with freshly prepared enzyme, using the fluorimetric method as modified by Adams [26] gave values for the liver enzyme ranging from 3.01 to 3.52 with an average value of 3.23. For the kidney enzyme the values ranged from 3.05 to 3.63 with an average value of 3.26. With preparations that had been stored at −15°C for varying periods of time, the values were lower, approaching 2 with older preparations. When B_6 content was determined by a microbiological assay [28] using either freshly prepared or stored enzyme, the average value was about two molecules of B_6 per molecule of enzyme as had been reported by others [12,16].

The possibility was investigated that the autoclaving procedure required for

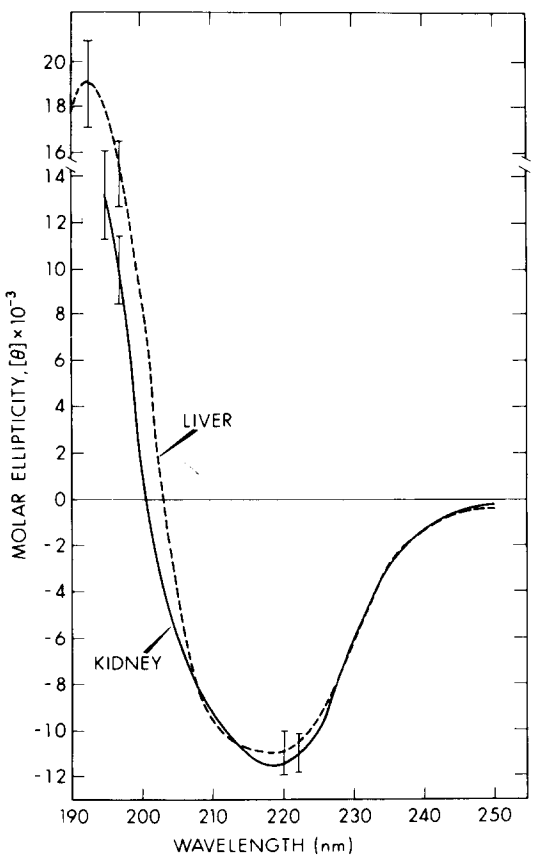

Fig. 6. Circular dichroism spectra of rat liver and kidney ornithine-oxo-acid aminotransferase. Protein concentrations were liver enzyme 115 μg/ml and kidney enzyme 165 μg/ml and a cell of 1 cm path length was used.

the microbiological assay was destructive of pyridoxal. Aliquots of each enzyme were autoclaved as required for the microbiological assay [28]. Pyridoxal then was determined fluorimetrically by the method of Toepfer et al. [27]. Parallel aliquots of each enzyme that had not been autoclaved were analyzed fluorimetrically for pyridoxal phosphate by the Adams procedures [26]. About 40% less pyridoxal was found in the solutions after autoclaving, suggesting that the coenzymes in these enzymes are altered or destroyed during heating. Instability of the coenzyme conceivably could account also for the loss seen during prolonged storage at −15°C. The average value of 3.2—3.3 molecules of coenzyme per molecule of enzyme is probably low since even the comparatively mild procedures of Adams [26] call for heating in acid solution at 50°C. Thus it seems reasonable to propose that the actual ratio of coenzyme to enzyme is 4 per 161 000 daltons or 1 per subunit. The decrease of measurable pyridoxal phosphate in stored enzyme samples is reflected by a loss of activity

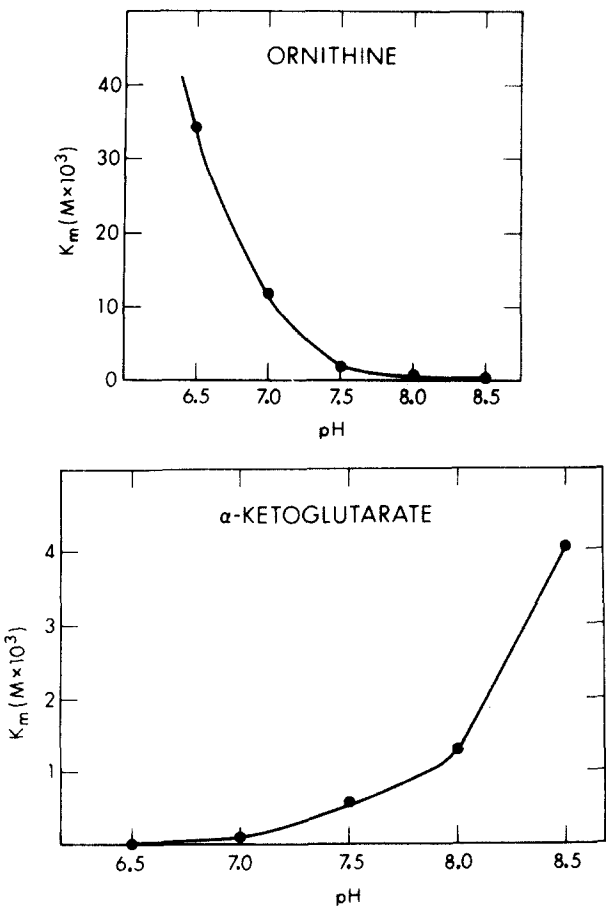

Fig. 7. Plot of K_m values for L-ornithine and α-ketoglutarate as a function of pH. Ornithine and α-ketogluarate concentrations were varied. All other conditions were as described under Methods. pH value of each incubation was adjusted initially by addition of KOH or HCl and redetermined at the end of the incubation.

of these samples that could be only partly restored by addition of pyridoxal phosphate. The saturating concentration of coenzyme for this restoration was 20 μg per ml. Preparations of enzymes in solutions containing pyridoxal phosphate, that were dialyzed or passed through Sephadex in order to remove exogenous pyridoxal phosphate, also yielded low values for bound coenzyme.

Absorption spectrum. The spectrum of liver and kidney ornithine-oxo-acid aminotransferases were determined over the wavelength range of 260—450 nm with α-ketoglutarate or ornithine added. As noted by others [12,14,16], two peaks were evident, one at 410—420 nm and the other at 320—330 nm. Work with other pyridoxal phosphate enzymes has established that the peaks in the 410 and 320 nm regions correspond to the pyridoxal and pyridoxamine forms respectively. The purified holoenzymes of either liver or kideny were isolated chiefly in the pyridoxal form which might be expected from the purification procedure which adds α-ketoglutarate at one stage. Peraino [38] has shown also that the pyridoxamine form of the liver enzyme partially reverts to the pyridoxal form. Thus it was to be expected that addition of α-ketoglutarate did not greatly increase the absorption at 410 nm. However, when compared to the kidney enzyme every preparation of liver enzyme either freshly prepared or stored at −15°C showed a lower specific absorption at 410 nm. Addition of excess α-ketoglutarate to either enzyme caused parallel increases of the absorption at 410 nm and decreases of the absorption at 330 nm. Conversely addition of L-ornithine to either enzyme caused parallel increases of the absorption at 330 nm and decreases of absorption at 410 nm for both enzymes. Circular dichroism spectra for the liver and kidney enzymes in the far ultraviolet region

TABLE I

INHIBITION OF KIDNEY ORNITHINE-OXO-ACID AMINOTRANSFERASE BY ANALOGUES OF L-ORNITHINE AND α-KETOGLUTARATE

Approx. 0.5 unit of kidney ornithine-oxo-acid aminotransferase was incubated as described in Methods with 50 μmol per ml of L-ornithine and 25 μmol of α-keto analogue inhibitor in each experiment except for DL-norvaline and L-canavanine of which 100 μmol and 20 μmol per ml, respectively, were added.

	Inhibition (%)
L-ornithine analogue	
D,L-Norvaline	44
L-valine	33
γ-Aminobutyrate	26
L-canavanine	23
L-Isoleucine	19
L-leucine	17
δ-Aminovalerate	15
ε-Aminocaproate	6
Cadaverine	5.7
Putrescine	3
α-Keto analogue	
Isocaproate	41
Valerate	38
Caproate	34
Butyrate	27
Isovalerate	25

are shown in Fig. 6. The calculated percentages of secondary structure of both liver and kidney ornithine-oxo-acid aminotransferases were 27, 11, and 62 for α, β and random forms within the limitation of the method [29]. It is noteworthy that significant amounts of β structure are present. The absorption of the bound pyridoxal phosphate contributed to the ellipticities in the visible and near ultraviolet regions where multiple ellipticity bands were seen that were not identical for the two enzymes. The phenomena accounting for these differences are being investigated.

Kinetic constants. The effect of pH on K_m values was examined by obtaining data for reciprocal plots of velocities versus substrate concentrations at pH values varying from 6.5 to 8.5. Fig. 7 presents data for ornithine that demonstrate that K_m which is relatively constant at pH 7.5, increased rapidly below this pH so that at pH 6.5 it was 17 times as high. The situation for α-ketoglutarate was reversed as shown also in Fig. 7. K_m rose rapidly as the pH was raised, being 25 times greater at pH 8.5 than at pH 7.0. Thus it appears that in the region of pH 7.0—7.5 the K_m values for both substrates are in their low ranges.

Inhibitors. A variety of compounds structurally related to L-ornithine previously shown to be inhibitory to liver ornithine-oxo-acid aminotransferase [1] was tested for their effects on the purified kidney enzyme. Table I presents the

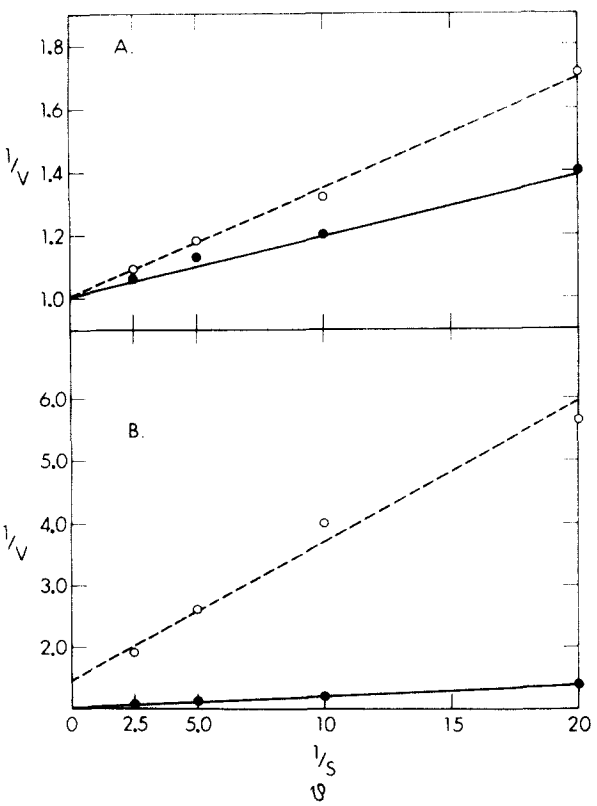

Fig. 8. A and B. Plots of reciprocal velocities against reciprocal ornithine concentrations. A, with L-canavanine added (20 mM) (○ - - - - - ○); B, with DL-norvaline added (50 mM) (○ - - - - - ○).

TABLE II

INACTIVATION BY HEAT AND CORRESPONDING CHANGES OF REACTIVE THIOL GROUPS OF LIVER AND KIDNEY ORNITHINE-OXO-ACID AMINOTRANSFERASES

Kidney or liver ornithine-oxo-acid aminotransferase, specific activity 1200—1500, in 0.18 M triethanolamine, HCl, 0.0045 M EDTA, pH 7.5, was maintained at 66—67°C. Aliquots containing 0.5 unit were removed at the times indicated for determination of enzyme activity. Parallel aliquots were removed, sufficient to yield absorptions of 0.4—0.6 absorbance units at 410 nm after titration with Nbs_2. Titrations were conducted in sodium dodecyl sulfate solutions as described in Methods.

Time (min)	Percent inactivated		Thiol content (mol/mol of enzyme)	
	Liver	Kidney	Liver	Kidney
0	0	0	16.20 ± 0.14	12.85 ± 0.13
15	95.9 ± 0.4	85.1 ± 1.1	15.24 ± 0.11	13.73 ± 0.08
30	97.6 ± 0.4	93.3 ± 0.4	12.03 ± 0.16	13.44 ± 0.25
60	98.1 ± 0.1	96.3 ± 0.1	11.02 ± 0.50	13.27 ± 0.26

relative inhibition measured with each of these compounds at similar concentrations. DL-Norvaline was the most potent inhibitor tested. This table also presents related data with analogues of α-ketoglutarate. In this series α-ketoisocaproate was most inhibitory. To determine the type of inhibition exerted on the kidney enzyme as compared with the results already obtained with the liver enzyme, the inhibitions exerted by DL-norvaline and L-canavanine were examined in greater detail. Fig. 8 shows the double reciprocal plots obtained with these two analogues of ornithine. It is seen that canavanine was competitive with ornithine whereas norvaline decreased V and increased K_m for the substrate. With both of these compounds similar findings had been made with the liver enzyme [1]. Nbs_2, *p*-chloromercuribenzoate ($ClHgBzO^-$) or iodoacetamide each react with SH groups of either the kidney or liver enzymes. Experiments with short time preincubations with these reagents demonstrated considerable difference in their effectiveness as inactivativators or inhibitors. Thus at $1 \cdot 10^{-2}$ M iodoacetamide, 15 min preincubation at pH 7.8 had little or no effect on enzyme activity. Under the same conditions $1.5 \cdot 10^{-2}$ M Nbs_2 or $1.4 \cdot 10^{-6}$ M $ClHgBzO^-$ inactivated either enzyme by 55—60 and 75—80%, respectively. The presence of substrates provided some protective effect. When

TABLE III

CYSTEINE CONTENT OF LIVER AND KIDNEY ORNITHINE-OXO-ACID AMINOTRANSFERASE

Reactions and determinations were conducted as described in Methods and in the text. At least five determinations were conducted for each procedure.

Method	Liver	Kidney
	(mol/mol enzyme)	(mol/mol enzyme)
Nbs_2 titration	16.20 ± 0.14	13.27 ± 0.13
Performic acid oxidation	16.63 ± 0.75	14.01 ± 0.34
Carboxymethylation	15.66 ± 0.24	13.96 ± 0.40

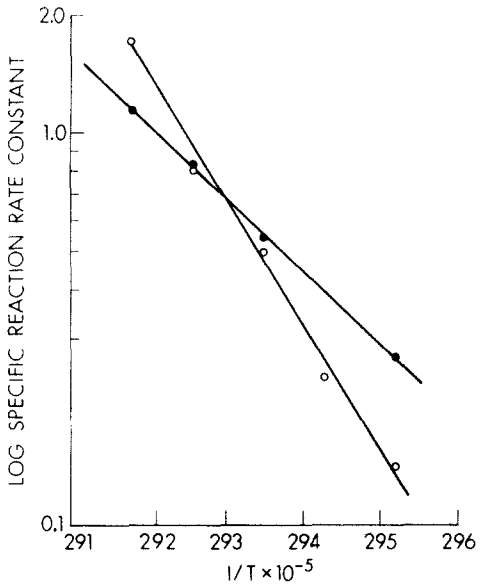

Fig. 9. Plot of first-order reaction rate constants for inactivation of liver and kidney ornithine-oxo-acid aminotransferase against reciprocals of absolute temperature. Solutions of the two enzymes were heated for periods up to 1 h at the temperatures indicated. Suitable aliquots were removed at 5—15 min intervals for determinations of residual enzymic activities. The first-order reaction rate constants were determined from the rates of inactivation. (○—○, liver ornithine-oxo-acid aminotransferase; ●—●, kidney ornithine-oxo-acid aminotransferase.)

ornithine and α-ketoglutarate were added before the thiol reagent the inactivations or inhibitions obtained were reduced to 15—20% with Nbs_2 and 55—60% with $ClHgBzO^-$.

Modification reactions. Attempts to modify liver and kidney ornithine-oxo-acid aminotransferases by reaction with maleic anhydride, succinic anhydride, or *N*-ethylmaleimide, by strict adherence to conditions described in the literature, led to incomplete reaction as judged by appearance of several bands of modified protein in subsequent electrophoresis on polyacrylamide gel. Under identical conditions of "incomplete reaction", fewer peptides were obtained with the liver enzyme suggesting that the two enzymes reacted at different rates with any given reagent. Alteration of the reaction conditions, by progressively increasing the concentrations of reagent and the reaction time finally resulted in acheiving a single peptide product as judged by gel electrophoresis. Optimal conditions in each case are described in Methods. In addition to the above-named reagents iodoacetate and acetic anhydride were used for modification. With each of the modified proteins, the corresponding products of the liver and kidney enzyme migrated at the same relative mobilities.

Thiol Content. Performic acid oxidation of liver and kidney ornithine-oxo-acid aminotransferases followed by hydrolysis and determination of cysteic acid [30,31] indicated that the liver enzyme contained at least two more cysteine residues per molecule of protein than did the kidney enzyme. The same difference was obtained by reaction with iodoacetate and determination of carboxymethyl cysteine. In a third approach both enzymes were titrated

TABLE IV

AMINO ACID COMPOSITION OF ORNITHINE-OXO-ACID AMINOTRANSFERASES FROM RAT LIVER AND KIDNEY RESIDUES PER 1000

Amino acid	Kidney		Liver	
	1	2	3	4
Leucine	101.20 ± 2.20	96	102	102.30 ± 2.50
Glutamic acid	100.36 ± 1.02	100	104	100.36 ± 1.11
Glycine	90.20 ± 2.01	87	90	87.90 ± 2.06
Aspartic acid	90.50 ± 0.76	88	93	88.50 ± 0.85
Alanine	83.32 ± 1.15	80	81	85.20 ± 1.05
Lysine	67.40 ± 0.38	60	58	68.6 ± 0.40
Valine	65.35 ± 0.85	68	72	62.76 ± 0.94
Proline	60.22 ± 0.75	60	61	60.40 ± 0.93
Isoleucine	53.42 ± 0.48	57	62	48.60 ± 0.55
Arginine	53.36 ± 1.25	47	52	53.46 ± 1.40
Threonine	52.28 ± 0.93	54	50	52.36 ± 0.92
Serine	48.40 ± 0.56	47	40	45.63 ± 0.61
Tyrosine	36.35 ± 0.20	37	40	42.96 ± 0.15
Phenylalanine	32.26 ± 0.38	31	32	32.16 ± 0.35
Histidine	24.50 ± 0.42	24	21	25.53 ± 0.50
Methionine	20.11 ± 0.85	36	16	19.96 ± 0.96
Tryptophan	14.20 ± 0.38	13.2	16	14.30 ± 0.30
Cysteine	11.22 ± 0.20	14	11	13.33 ± 0.15

with 5,5′-dithio-bis(2-nitrobenzoic acid)(Nbs_2). As previously reported for the liver enzyme, titration of native enzymes with Nbs_2 revealed only about 25% of the total SH groups. With sodium dodecyl sulfate added to a final concentration of 2%, the number of thiol groups titratable with Nbs_2 increased to the values shown in Table II. As presented in that table 2—3 more thiol groups were available in the liver enzyme. Thus by three methods the value for cysteine SH was higher for the liver enzyme as shown in Table III.

Heat stability. The observations that the liver and kidney enzyme appeared to differ in reactivity and stability prompted an examination of their heat stabilities. Solutions of the enzymes in dilute buffers of various pH values were maintained in a constant temperature bath. Aliquots were withdrawn at time intervals of 5—10 min over a period of 30 min and residual activity determined for each sample in a range of 55—65°C. At pH values up to 8.0 very little inactivation was obtained below 55°C. The inactivation rates, which were first order, increased as the temperature was raised, and decreased as the pH was lowered. At pH 5.9, over a temperature range of 65—70°C, the rates of inactivation could be conveniently determined by the method of assay. A plot of the logarithms of the specific first-order reaction rate constants of inactivation against the reciprocals of the absolute temperature is shown in Fig. 9. It may be seen that the slopes which are proportional to the activation energies of inactivation are quite different for the two enzymes. The energies of activation of inactivation for liver and kidney ornithin-oxo-acid aminotransferase, respectively, were calculated to be 146 500 and 81 500 cal per mol under these conditions.

Heat inactivation and thiol reactivity. The rates of inactivation at pH 7.5 were too rapid to be measured accurately enough to permit determination of

the specific reaction rate constants. However, heating at this pH was a useful approach to measuring changes in thiol content with concentrations of enzyme sufficiently high to be titrated with Nbs_2. Table II shows that in 15 min at 66°C, 96% of the liver enzyme was inactivated as compared to 85% of the kidney enzyme. During this period of heating, reactive thiol groups in the liver enzyme decreased by one whereas those in the kidney enzyme increased by one. Heating for an additional 45 min resulted in almost complete inactivation of either enzyme; and the number of Nbs_2-titratable thiol groups decreased by an additional 4 in the liver enzyme whereas there was no further change in reactive thiol groups of the kidney enzyme.

Amino acid composition. The amino acid composition of rat kidney ornithine-oxo-acid aminotransferase is presented in the first column of Table IV. The amino acid composition of the rat liver enzyme has been reported previously by two groups of investigators [14,15]. These data, recalculated to residues per 1000 total residues are shown in columns 2 and 3 of Table IV. The compositions of the two enzymes comparing these three analyses, were quite similar except for the values obtained for lysine, isoleucine, arginine, serine, tyrosine and methionine. The results of our amino acid analysis of liver enzyme are presented in the last column of Table IV; they show that liver and kidney enzymes, as prepared here, have compositions of close similarity except for content of tyrosine and perhaps isoleucine.

Discussion

Chemical and physical studies usually have revealed differences in catalytic and physical properties of isoenzymes that reflect non-identical primary structures [39], or conformational variations that could distinguish some isoenzymes with apparently the same primary structures [40].

Sanada et al. [12] have studied this enzyme as purified from liver, kidney and intestine of the rat. These investigators reported that the ultracentrifugal and electrophoretic behavior, absorption spectra, pH optima, K_m values for substrates, specificity and immunological gel diffusion analyses were similar for all three preparations. They concluded that the enzymes from all three organs were the same protein. Yip and Collins [13], in a somewhat parallel but less extensive study, came to the same conclusion for liver and kidney ornithine-oxo-acid aminotransferase.

The data reported in this study confirm some of the data reported by these investigators and also by Peraino et al. [14] obtained with the liver enzyme. But observations presented in this paper, while extending information about the two enzymes, more importantly provide significant evidence that the liver and kidney ornithine-oxo-acid aminotransferases are different proteins.

The molecular weight of both kidney and liver enzymes was found to be 160 000 by equilibrium sedimentation and showed no concentration dependence within the range of protein concentrations used. This value is in general agreement with that reported by others [8,12,14,16].

In the present studies, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of both the kidney and liver enzymes revealed single bands with the same mobilities corresponding to a molecular weight close to 43 000. This

value suggests that the native enzyme of molecular weight of 160 000—172 000 has four subunits, each of about 43 000 molecular weight.

The Ouchterlony double-diffusion patterns demonstrated closely similar immunological properties for the liver and kidney enzymes in confirmation of Sanada et al. [12].

Pyridoxal phosphate content of the two enzymes was also the same approaching a ratio of 4 per molecule of enzyme and suggests a 1 : 1 ratio for the subunits. Matsuzawa et al. [16] reported a value of about 2 for the pyridoxal phosphate content, but as shown here, the lower value was probably due to the instability of the coenzyme. The light absorption spectra for freshly prepared samples indicated that the kidney enzyme retained a greater fraction of the coenzyme in the aldehyde form. However, addition of an appropriate substrate appeared to bring either enzyme to the same ultimate level of pyrodoxamine or pyridoxal form.

Circular dichroism spectra in the far ultraviolet region were the same for both enzymes. K_m values of the kidney enzyme were altered significantly by the pH of the incubation mixture. This finding could explain discrepancies that are evident in comparing the values obtained in different laboratories. These kinetic constants were similar for liver and kidney ornithine-oxo-acid aminotransferases. Analogues of ornithine and of α-ketoglutarate inhibited the kidney enzyme as they had been reported previously to inhibit the liver enzyme [1]. Inactivation of both enzymes was obtained with thiol-reacting reagents.

Modification of both the liver or kidney ornithine-oxo-acid aminotransferases by reagents reacting with amino or sulfhydryl groups did not reveal any differences in subunits obtained subsequently.

The data thus far discussed are on the whole in agreement with the conclusions of other investigators that liver and kidney ornithine-oxo-acid aminotransferases are identical proteins.

However, it was noted that the liver enzyme reacted more rapidly with certain amino and sulfhydryl group reagents, that it was less stable to the purification procedure and to storage, and that it appeared to retain a somewhat lesser fraction of its coenzyme in the aldehyde form as opposed to the amine form.

Significant differences in the heat stabilities of the two enzymes were demonstrated in a range of temperatures above 55°C. At pH values near neutrality or higher, the liver enzyme was inactivated more rapidly than the kidney enzyme but the rates of inactivation were so rapid that reaction rate constants could not be measured accurately. Lowering the pH to 5.9 decreased the rates of inactivation so that more accurate measurements could be obtained. At this pH the kidney enzyme was more labile and a significant difference in energies of activation of inactivation was calculated.

It is possible that these differences in stabilities are related to thiol content. Titration of thiol groups of either native enzyme with Nbs_2 revealed only about 25% of the total titratable thiol content; this was found also by Peraino et al. [14] for the liver enzyme. Titration in the presence of sodium dodecyl sulfate demonstrated that the liver enzyme contained at least two more titratable groups than the kidney enzyme. That these values for thiol groups could

be ascribed to cysteine residues was confirmed by direct determination of cysteine as cysteic acid and as carboxymethyl cysteine. These thiol groups in the kidney enzyme were unaffected with respect to Nbs_2 titration after 1 h at 66—67°C during which period activity was almost completely lost. Under the same conditions the liver enzyme lost at least 30% of its SH groups perhaps due to oxidation. Although complete loss of enzymatic activity can take place without oxidation of sulfhydryl groups, one or more of these residues may be involved in the catalytic function since Nbs_2 and $ClHgBzO^-$ are inhibitory. It is of interest also that these enzymes contain all cysteine residues as such, without disulfide bridges; direct determination of cysteine residues gave the same values as obtained by Nbs_2 titration.

The amino acid compositions of the two enzymes otherwise are rather similar. Possible significant differences were obtained for tyrosine and isoleucine in addition to cysteine.

Thus, substantial evidence has been found showing that despite many catalytic and physical similarities, the ornithine-oxo-acid aminotransferases of liver and kidney do differ in primary structure, at least with regard to the number of cysteine residues and perhaps with regard to tyrosine and isoleucine. Peptide mapping and the elucidation of the primary sequences should define the differences more precisely. Such knowledge is essential for understanding the significance of structural alterations in ornithine-oxo-acid aminotransferases during differentiation of liver and kidney.

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