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Rat Kidney L-α-Hydroxy Acid Oxidase: Isolation of Enzyme with One Flavine Coenzyme per Two Subunits†

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ABSTRACT: L-α-Hydroxy acid oxidase (listed as EC 1.4.3.2, L-amino acid: O_2 oxidoreductase) has been purified 100-fold from rat kidney to apparent homogeneity by gel electrophoresis. A subunit molecular weight of 47,500 was found by sodium dodecyl sulfate gel electrophoresis, but in contrast to previous reports, the enzyme has been found to have a molecular weight of ca. 200,000 by Sephadex gel filtration and by dodecyl sulfate gel electrophoresis of the enzyme cross-linked with dimethyl suberimidate. A somewhat higher value was found by sedimentation equilibrium, but a tetrameric structure for the active enzyme is definitely established. The enzyme was found to contain the FMN coenzyme at a concentration of one FMN/102,000 daltons or one flavine/two subunits, a highly unusual finding. This ratio was determined from spectroscopic analysis of the FMN in lyophilized samples of the enzyme and by titration of the coenzyme with the flavine specific enzyme inactivator 2-hydoxy-3-butynoate. The enzyme has the same specific activity as a crystalline sample of the enzyme reported to have twice as much flavine/milligram.

In the course of investigations into flavoenzyme reaction mechanisms, we have recently been concerned with the proposal that flavine-linked enzymes which oxidize α-hydroxy acids or α-amino acids generate transient α-carbanionic species during catalysis (Walsh et al., 1971). These studies have involved the detection of flavoenzyme-catalyzed elimination reactions with β-halo substrates (Walsh et al., 1971, 1973) and the susceptibility of certain bacterial flavoproteins to irreversible inactivation by the acetylenic suicide substrate 2-hydroxy-3-butynoate (Walsh et al., 1972a,b; Lederer, 1974). To extend these studies, we wished to isolate the previously described rat kidney L-hydroxy acid oxidase (Blanchard et al., 1945, 1946; Nakano and Danowski, 1966; Nakano et al., 1967), an unusual enzyme in possessing comparable oxidase activity toward both hydroxy acids and amino acids.

After the isolation of this enzyme and purification to apparent homogeneity by published procedures (Nakano and Danowski, 1966; Nakano et al., 1967), physical character-

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ization prior to mechanistic studies revealed several properties different from previous reports. In particular, the enzyme has been reproducibly found to be a tetramer of very similar subunits and to contain two flavine coenzymes, i.e., one flavine/two subunits.

Experimental Procedure

Materials. Amino acids and hydroxy acids were obtained from the Sigma Chemical Co. and were used without further purification, except for sodium D,L-α-hydroxy-*n*-valerate, which was recrystallized from ethanol to remove a contaminant which inhibited L-hydroxy acid oxidase. β-Chlorolactate was purchased from Sigma and freed from oxalate by the procedure of Koelsch (1930). Glycolic acid and nitroethane were obtained from Aldrich. Coomassie Blue, phenazine methosulfate, nitroblue tetrazolium, and sodium dodecyl sulfate, which was recrystallized from ethanol, were obtained from Sigma. Methylenebisacrylamide, which was twice recrystallized from acetone, and electrophoresis grade acrylamide were supplied by Eastman; while Cyanogum 41 was a product of the E.C. Apparatus Corp. Bovine serum albumin, beef heart and rabbit muscle lactate dehydrogenases, phosphorylase *a*, trypsin, ovalbumin, alcohol dehydrogenase, chymotrypsinogen *a*, ribonuclease *a*, catalase, and egg white γ-globulin were purchased from Sigma, while creatine kinase and pyruvate kinase were supplied from Boehringer, Mannheim. [^{14}C]Serine was from New England Nuclear. Ammonium sulfate was "ultrapure" from Schwarz/Mann; EDTA, NAD^+, NADH, and FMN were from Sigma. 2,6-Dichloroindophenol was obtained from Fisher and potassium ferricyanide from Mallinckrodt. All other inorganic chemicals were reagent grade from various suppliers. Blue Dextran 2000 and Sephadex gels were supplied by Pharmacia, and DEAE-cellulose was supplied by Whatman.

Determination of Protein Concentration and Enzyme Activity. Protein concentrations were determined by the spectrophotometric procedure of Warburg and Christian (1941). For enzyme purified through step 5 of Table I, the protein concentration was obtained from the absorbance at 280 nm multiplied by 0.88, a correction factor determined from the absorbance of a weighed sample of desalted, lyophilized enzyme purified through step 6 of Table I. Enzyme so treated retained 97–100% of its enzymatic activity. Protein assay by the procedure of Lowry et al. (1951) with bovine serum albumin gave values that were 80% of the protein concentration determined directly from dry weight, and was not generally used.

Unless otherwise noted, enzyme assays were performed at 25°. The standard activity assay solution consisted of 25 m*M* D,L-α-hydroxybutyrate, 0.002% DCIP,[1] enzyme, and 0.1 *M* sodium phosphate with 1 m*M* EDTA at pH 8.4 to a final volume of 1.0 ml. The reaction was continuously monitored in a Gilford Model 222 spectrophotometer at 600 nm. An extinction coefficient of 22,000 was used for DCIP at 600 nm at pH 8.0 (Armstrong, 1964). In some cases, potassium ferricyanide, extinction coefficient 1040 at 420 nm, replaced DCIP. Oxidase activity was measured with a 0.4-ml sample volume Clark-type oxygen electrode and a Keithley 4145 picoammeter. Production of keto acids was followed by a 2,4-dinitrophenylhydrazine assay (Bohme and Winkler, 1954) or by a coupled assay with rabbit muscle lactate dehydrogenase and NADH. All enzyme activity values in this paper refer to the DCIP procedure. The ferricyanide assay gave values 100–115% of those of the DCIP assay, and the oxygen electrode assay in the presence of catalase in air saturated solution gave results which were 65–70% of the expected values (half the DCIP rate). The enzyme activity unit is nanomoles of dye reduced/minute; specific activity is given as units/milligram of protein.

[1] Abbreviation used is: DCIP, 2,6-dichloroindophenol.

Enzyme Purification

Soluble Enzyme. Rat kidneys were obtained from Pel-Freez Biologicals, Inc., as fresh or frozen tissue; they were also prepared fresh from rats of mixed sex after decapitation. No apparent differences in the amount or properties of the enzyme from these sources were found. All purification and storage of the enzyme was done at 0–3°. The kidneys were cleared of membranes and fat and homogenized in 0.25 *M* sucrose–10 m*M* Tris-HCl at pH 8.0 (4 ml/g of tissue) in one of two ways. In early experiments the kidneys were disrupted for three 30-sec periods at high speed in a Waring Blendor. In other experiments (see Results) several passes in a motor driven Thomas Teflon pestle tissue homogenizer were used for cell disruption. Cell debris was removed by centrifugation at 1500*g* for 10 min. The supernatant was centrifuged at 8500*g* for 10 min to separate the mitochondria. In view of the report that the soluble and mitochondrial oxidases of rat kidney have different electrophoretic mobility (see Discussion), the mitochondrial pellets from several preparations were combined and purified separately from the soluble enzyme. These experiments are described after the purification of the enzyme from the soluble fraction has been detailed. After the supernatant from the last centrifugation had been recentrifuged at 18,000*g* for 30 min, the soluble fraction was obtained. The enzyme activity at this stage could only be determined after passage of a sample through Sephadex G-25 to remove species (presumably thiols) which interfered with the dye assay.

The soluble fraction was taken to 32% saturation of ammonium sulfate and the pH adjusted to 8 with NaOH. The precipitate was isolated by centrifugation and dissolved in 5 m*M* sodium phosphate–0.5 m*M* EDTA (3 ml/g of precipitate) at pH 7. During dialysis against 2 × 4 l. of the same buffer a large amount of denatured protein appeared in the dialysis sacs. This protein was removed by centrifugation before enzyme activity assay.

The supernatant from the previous step was applied to a 5 × 15 cm DEAE column equilibrated with 5 m*M* sodium phosphate at pH 7. After the protein had been applied the column was washed with 500 ml of this buffer and then developed with ca. 1 l. of 50 m*M* sodium phosphate at pH 8. Eluent was collected until a 0.5-ml sample showed no enzyme activity in the standard assay.

The pH of the column elutant was adjusted to pH 8 and taken to 35% saturation of ammonium sulfate. The precipitate was removed by centrifugation and the supernatant treated with ammonium sulfate to 50% saturation and stirred for 1 hr. After centrifugation, the pellet was dissolved in 10 m*M* sodium phosphate and 1 m*M* EDTA at pH 7 and exhaustively dialyzed against this buffer. Denatured protein which formed was removed by centrifugation.

The dialysate was applied to a 4 × 15 cm column of DEAE and equilibrated with the buffer of the previous paragraph. The loaded column was washed with 500 ml of the equilibration buffer and then developed with a linear gradient between 400 ml of the equilibration buffer and 400 ml of 50 m*M* sodium phosphate–1 m*M* EDTA at pH 8.4.

During the development a bright yellow band separated from the reddish brown protein at the top of the column and passed along the column. Fractions of 10 ml volume were collected during the elution of this yellow protein and all those fractions having a specific activity greater than 200 nmol per min per mg were pooled. The enzyme was concentrated by addition of ammonium sulfate to 50% saturation, centrifugation at 18,000*g* for 30 min, and dialysis against 500 volumes of 50 m*M* sodium phosphate–1 m*M* EDTA at pH 8.4.

The light yellow enzyme solution was applied to a 6 × 110 cm column of Sephadex G-100 equilibrated with 50 m*M* sodium phosphate–1 m*M* EDTA at pH 8.4. The enzyme eluted from this column in a symmetrical peak immediately behind the void volume. The enzyme was concentrated with ammonium sulfate to 50% saturation and dialysis as in the previous paragraph.

In some cases (discussed in the Results section) isozymes were separated by another DEAE column. Enzyme from the previous step was dialyzed against 10 m*M* sodium phosphate at pH 7.5. A 1.5 × 5 cm column of DEAE was equilibrated with this buffer, the enzyme applied, and the enzyme developed with a gradient between 100 ml of this buffer and 100 ml of 10 m*M* sodium phosphate at pH 7.5 containing 100 m*M* potassium chloride. Although essentially no increase in specific activity was obtained, the last enzymatically active fractions contained only the major isozyme.

Mitochondrial Enzymes. The mitochondrial fractions were disrupted by two freeze–thaw cycles and mixed with three volumes of 10 m*M* sodium phosphate at pH 7. After the mixture was stirred for 1 hr the suspension was centrifuged at 25,000*g* for 2 hr to afford the enzyme solubilized from mitochondrial fraction. The activity in this crude supernatant was then isolated by the procedure used for the soluble enzyme with the following exception. The enzyme applied to the first DEAE column was eluted with a linear gradient between 5 and 200 m*M* sodium phosphate at pH 7.5. The visibly yellow fractions which eluted were combined and further purified by the procedure given for the soluble enzyme. Unlike the soluble enzyme purification, the mitochondrial enzyme as isolated showed no evidence for isozymes and a final DEAE column was not required.

Electrophoresis. Analytical disc polyacrylamide gel electrophoresis was conducted after Davis (1964) at 4° in 12-cm gels. The running gels were prepared from 6.25% Cyanogum 41, and no stacking gels were used. Gels were stained for protein with Coomassie Blue in 10% acetic acid solution containing 25% by volume isopropyl alcohol and destained in 7.5% acetic acid. Staining for enzyme activity was performed as described by Feinstein and Lindahl (1973); optimum results were obtained with a staining solution containing 1 mg of nitroblue tetrazolium, 5 m*M* substrate to be investigated, and 15 ml of 0.05 *M* sodium phosphate buffer at pH 8.4. The activity stain gels were incubated at 37° in the dark until the activity bands were clearly visible.

Subunit molecular weights were determined by the sodium dodecyl sulfate procedure of Weber and Osborn (1969) except that 7.5% acrylamide gels with either 0.03 or 0.02 ratio of methylenebisacrylamide to acrylamide were used. The gels employed were 12 cm in length and were destained and stored in 12.5% trichloroacetic acid solutions.

Urea gels were prepared as described above for the analytical disc gel electrophoresis gels with the addition of 8 *M* urea and use of sufficient Cyanogum 41 to give either 3 or 4% gels. Enzyme samples were denatured in 8 *M* urea containing 1% β-mercaptoethanol for 10 hr at 4° before application to gels. After the electrophoresis apparatus had been prepared but before the addition of sample to the top of the gels, it was necessary to fill the space above the gels with tray buffer containing 4 *M* urea. If the tray buffer was used directly, the enzyme tended to precipitate at the gel interface and not enter the gel. Staining was conducted with Coomassie Blue in 7.5% acetic acid as described above.

Molecular Weight Determinations by Sephadex Gel Chromatography and Sedimentation Equilibrium. A 120 × 2 cm Sephadex G-200 column was equilibrated with 0.1 *M* sodium phosphate at pH 7 and was calibrated for molecular weight determination according to Andrews (1964). The void volume (28 ml) was measured with Blue Dextran 2000 and the final elution volume (73 ml) with [^{14}C]-L-serine. Protein was measured spectrophotometrically at 280 or 235 nm.

Sedimentation equilibrium experiments were conducted at 5° in a Beckman Model E centrifuge using interference optics. The enzyme concentration was 0.74 mg/ml in a buffer of 0.1 *M* KCl and 50 m*M* sodium phosphate at pH 8.4. Fringe displacements were measured after 18 hr at 12,000*g*. Molecular weights were plotted as a function of fringe displacement and extrapolated to zero fringe displacement. No evidence for protein association was found. For the molecular weight calculations a value of 0.74 was assumed for the partial specific volume of the protein and a solution density of 1.000 was used.

Cross-Linking of Subunits with Dimethyl Suberimidate. To 50 μl of 0.5 *M* hydroxybutyrate and 0.5 mg of dimethyl suberimidate adjusted to pH 9 at 0° was added 375 μg of enzyme in 75 μl of 0.1 *M* sodium phosphate buffer. Half of this solution was added to 50 μl of the protein denaturing buffer of Weber and Osborn (1969) immediately and the other half after 7 hr. After denaturation for 4 hr at 37°, the samples were run on the sodium dodecyl sulfate acrylamide gels described by Lad and Hammes (1974). Electrophoresis was carried out at 10 mA/tube. The gels were stained in Coomassie Blue, and the relative mobility of the protein bands determined. Phosphorylase *a* was also run as a molecular weight marker.

Results

Purification and Stability of the Enzyme. The original intent of this work was to isolate the soluble L-hydroxy acid oxidase (described initially as L-amino acid oxidase) of rat kidney described by Nakano et al. (1967). Consequently, the purification procedure of those workers was closely followed. However, in our hands the enzyme showed much reduced affinity for DEAE-cellulose at the pH and ionic strength reported by them. Satisfactory purification of the enzymatic activity in our experiments on DEAE columns could be achieved at a lower pH and a lower ionic strength. The purification protocol described in Table I afforded the same enzyme from either fresh or frozen rat kidneys and from rat kidneys obtained commercially from Pel-Freez Biologicals or from rats decapitated in this laboratory. Furthermore, enzyme of identical electrophoretic mobility, catalytic properties, and coenzyme content was isolated from the mitochondrial and the soluble fractions of rat kidney cells, in contrast to the observations of Domenech et al. (1973), who found electrophoretically distinct isoenzymes in the mitochondrial and soluble fractions.

The L-hydroxy acid oxidase of Table I was observed to be

Table I: Summary of Purification of Oxidase from Rat Kidney.

Stage	Fraction	Volume (ml)	Protein[a] (mg/ml)	Protein[a] (Total)	Enzyme Activity[b] (Specific)	Enzyme Activity[b] (Total)	Recovery (%)
Enzyme from soluble fraction							
1	Soluble fraction	450	29.5	13,300	5.42	72,000	
2	First $AmSO_4$ precipitation	72	33.7	2,400	22.2	54,000	75
3	First DEAE column	244	5.84	1,400	40.2	48,000	67
4	Second $AmSO_4$ precipitation	47	15.8	743	50.4	37,000	51
5	Second DEAE column and third $AmSO_4$ precipitation	15	5.27	79	285	22,000	31
6	Sephadex G-100 column	8.8	4.59	40	416	16,600	24
Enzyme from mitochondrial fraction							
1	Crude supernatant	1750	7.12	12,500	4.13	51,500	
2	First $AmSO_4$ precipitation	94	19.3	1,800	27.7	50,300	98
3	First DEAE column	250	2.19	548	64	35,100	68
4	Second $AmSO_4$ precipitation	24	15.9	382	76	29,000	56
5	Second DEAE column and third $AmSO_4$ precipitation	8	8.24	65.9	298	19,600	38
6	Sephadex G-100 column	8	3.43	27.4	450	12,300	24

[a] By method of Warburg and Christian (1941). [b] Assayed with 25 m*M* D,L-α-hydroxybutyrate as described in the text.

stable in solution at 4° in the dark for several months. It could be dialyzed against 2 *M* potassium bromide for 2 days without loss of activity (Massey and Curti, 1966), suggesting tight binding of the coenzyme, and 97–100% of the activity was retained after lyophilization and redissolution. The enzyme was capable of oxidizing poor substrates at a constant V_{max} for 24 hr at 25° but was found to readily denature in acid.

Table I shows a purification of 77-fold for the enzyme from the soluble fraction and 110-fold from the crude mitochondrial fraction. In both cases, the enzyme eluted from the Sephadex G-100 column (step 6 of Table I) in a symmetric band with specific activity of 365–476 for the soluble enzyme and 450 for the mitochondrial enzyme.

The results of disc gel electrophoresis with stains both for protein and for enzymatic activity on both the soluble and mitochondrial enzymes are presented in Figure 1. Gel A is the soluble enzyme purified through step 6 of Table I and stained with Coomassie Blue. Of the four discernible bands in this gel three were demonstrated to have hydroxy acid oxidase activity by staining a similar gel (Gel B) for enzyme activity toward D,L-α-hydroxybutyrate (Feinstein and Lindahl, 1973). An identical pattern was obtained when the soluble enzyme was stained for enzymatic activity toward L-leucine (not shown). By densitometry, the major band was found to represent 77% of the total activity stain. After chromatography on a third DEAE column (see Experimental Procedures) to remove the minor isoenzymes, however, the soluble enzyme stained as a single band with Coomassie Blue (gel C) or by either α-hydroxybutyrate (gel D) or L-leucine (gel E) activity stains. The mitochondrial enzyme stained as a single band with Coomassie Blue (gel F) or either of the activity stains (not shown, for they were indistinguishable from the corresponding stains for the soluble enzyme), after passage through the Sephadex G-100 column. Gel G is an equal mixture of the soluble and mitochondrial enzymes stained with Coomassie Blue. Since either hydroxy acid or amino acid activity stains also failed to demonstrate any difference in the two enzyme fractions, we conclude that in each case a single protein was obtained and that these proteins are electrophoretically indistinguishable. Although the gels of Figure 1 are of different lengths, the major band in each has an identical R_f of 0.33. Gel C is heavily overloaded; only one band was also found on gels with less protein, and the protein band was as sharp as gels C and F. The division into mitochondrial and soluble fractions is a technical designation since in vivo enzyme is probably concentrated in the peroxisomes of rat kidney (McGroarty et al., 1974).

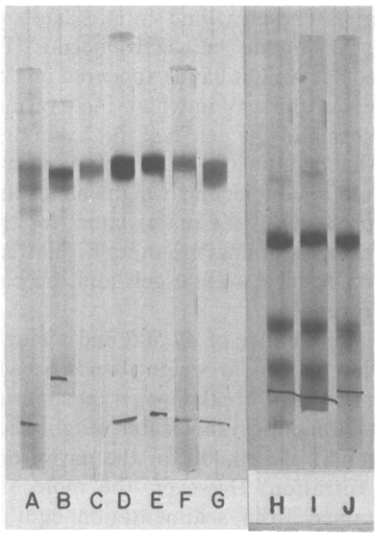

FIGURE 1: Polyacrylamide gel electrophoresis of L-α-hydroxy acid oxidase of rat kidney. (A, B) Soluble enzyme after Sephadex G-100 chromatography; (C–E) soluble enzyme after a third DEAE column (see text); (F) mitochondrial enzyme after Sephadex G-100 chromatography; (G) a mixture of equivalent amounts of the mitochondrial and the soluble enzyme. (H–J) sodium dodecyl sulfate polyacrylamide gel electrophresis (H, soluble enzymes; I, mitochondrial enzyme; J, mixture of soluble and mitochondrial enzymes). Gels A, C, F–J were stained with Coomassie Blue while B, D, and E were stained for oxidase activity toward D,L-α-hydroxybutyrate (B,D) or L-leucine (E).

Molecular Weight and Subunit Structure. In the concentration range of microgram per milliliter L-α-hydroxy acid oxidase appears to have a molecular weight of 190,000 as judged by its behavior on Sephadex G-200 gel exclusion chromatography. Such Sephadex chromatography is known to provide the Stokes radius directly and the molecular weight only when the standard proteins and the protein of interest have similar shapes in solution (Determann and

Michel, 1966). In contrast to the value of 190,000 obtained by gel filtration, a value of 250,000 was found by sedimentation equilibrium experiments. There was no indication of protein self-association in these experiments. At the present time we have no explanation for the discrepancy in molecular weight although it may point out that the oxidase exhibits a nonglobular shape in solution.

The subunit composition of L-α-hydroxy acid oxidase was determined by dodecyl sulfate gel electrophoresis (Figure 1) in the usual manner (Weber and Osborn, 1969). Subunit molecular weight determination was carried out in gels with differing degrees of cross-linking with the result that the major band from both the soluble and the mitochondrial preparations was about 47,500. Furthermore, no evidence for differences in subunit size for this major band could be found in either case (Domenech et al., 1973). The subunit size of this multimeric enzyme had not been previously determined. The sodium dodecyl sulfate gels of Figure 1 also show the presence of two lower molecular weight bands, one at 21,000 and one at 17,000. These gels were overloaded to show these lower molecular weight bands clearly. At normal protein concentrations these bands were visible but faint. These enzyme samples had shown only one band on polyacrylamide gel electrophoresis (Figure 1), but the low molecular weight bands appeared on dodecyl sulfate gels regardless of the way in which the enzyme was treated before application of the gel [boil for 2 min or heat for 8 hr at 46° in the denaturing buffer of Weber and Osborn (1969)]. These low molecular weight bands are apparently breakdown products of the major band, for they appear in dodecyl sulfate gels of enzyme samples that have been previously run on dodecyl sulfate gels and the band at 47,500 excised and rerun.

From the subunit size of 47,500 and assuming the small amounts of low molecular weight bands on dodecyl sulfate gels represent subunit breakdown, a tetrameric structure of four subunits of identical molecular weight accounts for the molecular weight of 190,000 for the native enzyme by Sephadex gel filtration. However, the molecular weight of 250,000 obtained from sedimentation equilibrium would suggest either a pentameric structure or a tetramer with associated smaller fragments. The breakdown studies of the previous paragraph eliminate the latter and protein cross-linking experiments eliminate the former. When the enzyme is incubated for long times (7 hr) with the cross-linking reagent dimethyl suberimidate (Davies and Stark, 1970), and then run on the dodecyl sulfate gels of Lad and Hammes (1974), only three bands appear. The lowest molecular weight band is the monomer (comparison of position with uncross-linked enzyme) at a relative mobility of 0.92. A very light band at a relative mobility of 0.56 was assigned to the dimer (mol wt ~95,000) since phosphorylase *a* (mol wt 94,100) ran at an almost identical position in an identical gel. The heaviest band in the gel had a relative mobility of 0.145, a value that gives it a molecular weight of 210,000 by extrapolation. No evidence for a trimer or any higher molecular weight species than the tetramer was observed.

When the enzyme was subjected to electrophoresis in gels containing 8 *M* urea, two distinct bands were observed. In 3% acrylamide gels these bands had R_f's of 0.22 and 0.30 while values of 0.12 and 0.21 were observed in 4% acrylamide gels. The bands were about equally intense and no fast running bands such as seen in the dodecyl sulfate gels were found. This observation suggests that there may be two kinds of subunits with differing charge which constitute the enzyme. On the basis of all the above evidence, we conclude that the active enzyme is a tetramer of subunits with very similar molecular weights but two different charge types. The arrangement of flavine among these subunits has not yet been determined. Still further evidence for this structural arrangement was found in the inactivation of the enzyme by 2-hydroxy-3-butynoate discussed later.

Identification and Content of Coenzyme. The coenzyme of L-hydroxy acid oxidase from the soluble fraction of rat kidney cells has earlier been identified as FMN. We confirmed this for our enzyme preparations by comparison of the relative mobility of the coenzyme on silica gel in the solvent system of Fazekas and Kokai (1971) with authentic samples of FMN and FAD. The coenzyme was liberated either by boiling the enzyme, by treatment of the enzyme with trichloroacetic acid at 4°, or by the procedure of Blanchard et al. (1945). When FAD was carried through these procedures, less than 10% breakdown, as judged by loss of fluorescence after thin-layer chromatography as described, was observed.

During the later stages of purification of all preparations, the visible yellow color of the hydroxy acid oxidase seemed low compared to other flavoenzymes we have previously isolated which have the same subunit size (e.g., D-amino acid oxidase, lactate oxidase) and presumably about the same amount of flavine coenzyme per milligram of enzyme protein. Despite the fact it has been reported (Nakano and Danowski, 1966) that the enzyme contains one FMN/49,300 daltons, we were prompted to reinvestigate the flavine content. The percentage of FMN in the enzyme was determined by treating a known weight of enzyme with 12.5% trichloroacetic acid at 0° to remove all the flavine from the protein (all 450 absorbance removed). The denatured enzyme was removed by centrifugation and the pH of the supernatant adjusted to 7. The absorbance of this solution at 450 nm was measured against a blank of 1 mg/ml of bovine serum albumin that had been identically treated. An extinction coefficient of 12,200 was used to determine the concentration of the coenzyme (Whitby, 1953). When this experiment was carried out on three different purified enzyme samples from rat kidneys that had been disrupted with a Waring Blendor, values of 4.47, 4.84, and 4.72 μg of flavine/mg of enzyme were obtained. Identical results were obtained when the enzyme was desalted by Sephadex filtration or by exhaustive dialysis against distilled water and then was lyophilized (to give the dry weight of the enzyme directly). No coenzyme is lost by these measures since the lyophilized enzyme retained 97–100% activity if redissolved. The average value of 4.68 μg of FMN/mg of enzyme corresponds to one FMN/102,000 daltons of the enzyme, a value of FMN one-half of that reported by previous workers. Since the enzyme subunit size is 47,500, one molecule of FMN is present for every two subunits in the homogeneous enzyme. Then two molecules of flavine must be in the native tetramer, a point confirmed by the active site labeling data discussed later.

To rule out the possibility that some coenzyme had been lost during the cell disruption with the Waring Blendor or in the subsequent purification, the soluble enzyme was isolated using a more gentle cell disruption with a motor driven tissue homogenizer and a 5 μM concentration of FMN maintained in all purification steps, including dialyses. When the flavine content of this preparation was determined after removal of the external FMN during the Sephadex G-100 purification, a value of 5.16 μg of FMN/mg

Table II: Kinetic Data for L-α-Hydroxy Acid Oxidase at 25°.

	K_m (mM) Soluble	V_{max} (% of Hydroxybutyrate Oxidation) Soluble[a]
Hydroxy acid substrates		
D,L-α-Hydroxybutyrate	14	100
D,L-α-Hydroxyvalerate	13	62
D,L-α-Hydroxyisovalerate	8	13
D,L-α-Phenyllactate	71	1.5
D,L-Lactate	27	37
L-α-Hydroxyphenyllactate	1.9	87
D,L-α-Hydroxycaproate	3.2	62
D,L-β-Chlorolactate	28	161
Amino acid Substrates		
L-Leucine	15	3
L-Tryptophan	40	2.2
L-Methionine	53	5.4
L-Lysine	90	0.4
D,L-α-Aminobutyrate		0
Other substrates		
Nitroethane		0
Glycolate		0

[a] V_{max} for D,L-α-hydroxybutyrate = 714 nmol per min per mg.

of enzyme was obtained. Although slightly higher than before, this value is still consistent with an FMN content of two per tetramer.

pH Optimum and Substrate Specificity. Since the enzyme as we have isolated it contains only half as much flavine per milligram of protein as previously reported, it was of interest to determine the catalytic properties of the enzyme to compare with those reported by Nakano et al. (1967). The pH optimum for both D,L-α-hydroxybutyrate and L-methionine oxidation by the soluble enzyme was found to be 8.4 and all assays for enzyme activity were conducted at this pH. For both kinds of substrates, half the maximum rate was observed at pH 9.0 and pH 7.5. The substrate specificities for the soluble enzyme toward hydroxy acids and amino acids are given in Table II and are essentially identical for a preparation from the mitochondrial fraction. The V_{max} is given as a percentage of the rate of oxidation of D,L-α-hydroxybutyrate. The enzyme exhibits a preference for long chain substrates and is clearly a more efficient hydroxy acid oxidase than an amino acid oxidase. Quantitatively and qualitatively the substrate specificity reported for the enzyme by Nakano et al. (1967) is mimicked in our hands. One slight difference is in the ratio of turnover numbers for D,L-α-hydroxybutyrate to L-leucine. We find a ratio of 33:1 while a value of 21:1 can be calculated from previous work (Nakano and Danowski, 1966).

More importantly an oxidation rate of 442 nmol per min per mg for 12.5 mM D,L-α-hydroxyvalerate at 37° using a DCIP assay can be calculated for the crystalline soluble enzyme reported by Nakano et al. (1967). Under identical conditions we observe a rate of 462 nmol per min per mg. Thus our preparations of L-α-hydroxy acid oxidase appear to be fully enzymatically active milligram for milligram, even though they contain only half as much FMN/milligram of protein as the preparations of Nakano et al. (1967).

Domenech et al. (1973) have reported that α-keto acids are noncompetitive inhibitors of the oxidation of D,L-α-hydroxyisocaproate by an L-hydroxy acid oxidase isolated from rat kidney. The L-hydroxy acid oxidase reported in this paper showed completely competitive inhibition of lactate oxidation by pyruvate. Futhermore, it was found that

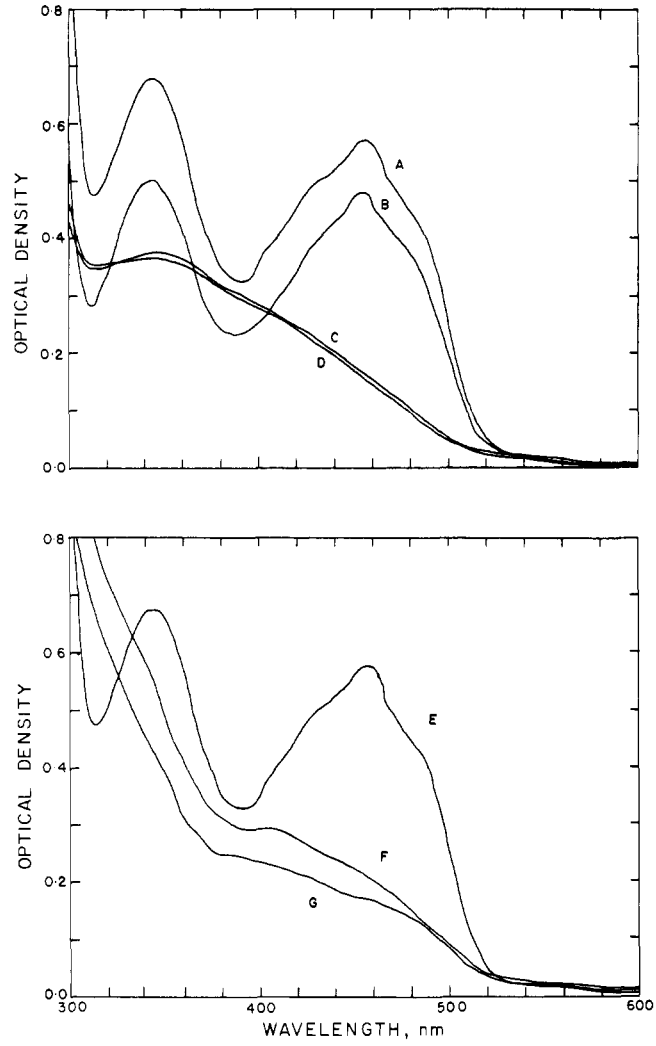

FIGURE 2: Absorption spectra of the soluble and mitrochondrial L-α-hydroxy acid oxidases. (A) Soluble enzyme at 4.6 mg/ml; (B) mitochondrial enzyme at 3.4 mg/ml; (C) soluble enzyme after anaerobic reaction with D,L-α-hydroxybutyrate; (D) soluble enzyme after anaerobic reaction with L-leucine; (E) soluble enzyme at 3.4 mg/ml; (F) soluble enzyme after reaction with dithionite; (G) soluble enzyme after reaction with sulfite.

the oxidation of D,L-α-hydroxybutyrate by the L-hydroxy acid oxidase described in this paper is competitively inhibited by L-leucine with a K_i of 23 mM (method of Dixon, 1953) as one would expect if both substrate types bind and are oxidized at the same active site.

Spectroscopic Properties of the Enzyme. The spectra of the soluble and mitochondrial L-hydroxy acid oxidases are presented in Figure 2. These spectra are unusual in that the lower of the two absorbance peaks characteristic of the flavine chromophore, which is generally found in the 360–390-nm range, is further blue shifted to 345 nm. Spinach glycolic acid oxidase has been shown to have this band at the still lower value of 340 nm (Frigerio and Harbury, 1958). For free FMN this lower wavelength band is found at 375 nm (Whitby, 1953). It should be noted that the rat kidney enzyme reported by Blanchard et al. (1946) and by Nakano and Danowski (1966) has this absorbance at 358 nm.

Under anaerobic conditions, the addition of the substrates D,L-α-hydroxybutyrate or L-leucine to the enzyme produced the reduced flavine spectra, curves C and D, respectively. The total loss of oxidized flavine absorbance on

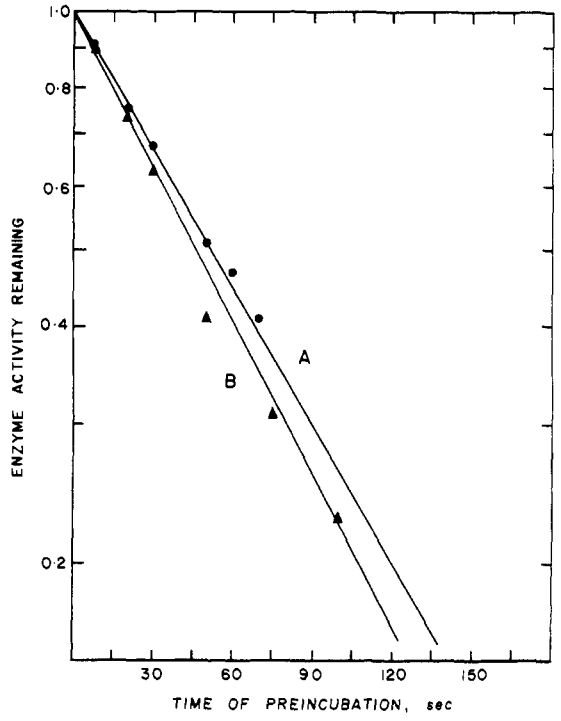

FIGURE 3: Loss of hydroxy acid and amino acid oxidase activity on reaction with D,L-2-hydroxy-3-butynoate. For line A, 24 mg of the soluble L-α-hydroxy acid oxidase was incubated with 1.67 mM D,L-2-hydroxy-3-butynoate and excess DCIP at 25° in a total volume of 60 μl of 50 mM sodium phosphate (pH 8.4). At the indicated times, 940 μl of 50 mM sodium phosphate at pH 8.4, containing 25 mM D,L-α-hydroxybutyrate was added, and the initial rate of dye reduction measured. The ordinate as given as the ratio of the enzyme activity after the given time of preincubation with D,L-α-hydroxy-3-butynoate to the enzyme activity after immediate quench. Line A was similarly constructed except that the initial preincubation mixture contained 120 μg of enzyme and 8.35 mM D,L-2-hydroxy-3-butynoate. In addition, the quench solution contained 50 mM L-leucine.

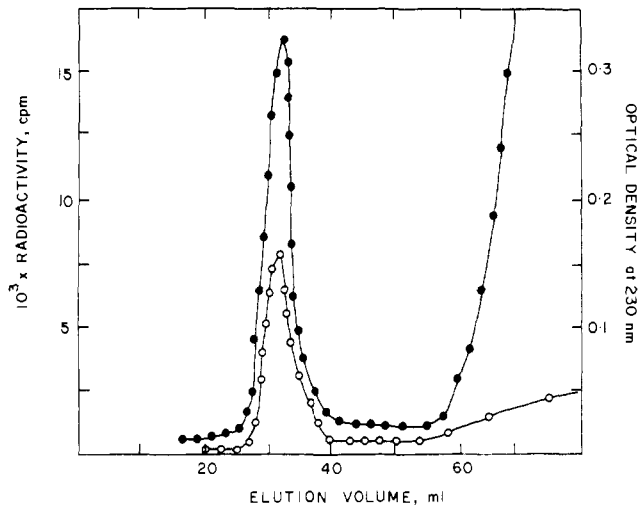

FIGURE 4: Sephadex G-200 gel filtration of L-α-hydroxy acid oxidase inactivated with [1-^{14}C]hydroxybutynoate; 0.5 mg of L-α-hydroxy acid oxidase was incubated with 0.5 μmol of [1-^{14}C]hydroxybutynoate (35 Ci/mol) in the presence of 2 mM dithiothreitol in 0.5 M sodium phosphate (pH 8.4). After 10 min at room temperature, the enzyme was applied to a Sephadex G-200 column previously described and equilibrated with 0.05 M sodium phosphate at pH 8.4. The radioactivity of the effluent (●) was determined by scintillation counting, and the protein measured spectroscopically at 230 nm (○).

the anaerobic addition of L-leucine confirms that the amino acid oxidase activity of L-hydroxy acid oxidase is an intrinsic property of the enzyme and is not due to a small amount of contaminating protein.

As shown in Figure 2, the flavine absorbance of the enzyme is also bleached by the addition of dithionite (curve F) or by sodium sulfite, (curve G) consistent with the findings of Massey et al. (1969) that flavoprotein oxidases are reduced by sulfite but that flavoprotein dehydrogenases are not.

Determination of FMN Content by [^{14}C]Hydroxybutynoate. The acetylenic hydroxy acid 2-hydroxy-3-butynoate has recently proven useful as a suicide substrate for the irreversible inactivation of certain microbial hydroxy acid oxidases and dehydrogenases, specifically those which require flavine coenzymes (Walsh et al., 1972a,b). Loss of enzymatic activity in those instances proceeds by covalent modification of only the bound coenzyme and consequently the enzyme can be used as a titrant for flavine coenzyme stoichiometry in susceptible enzymes.

As will be discussed in detail elsewhere (Walsh and Cromartie, to be published) hydroxybutynoate is also a suicide substrate for rat kidney L-hydroxy acid oxidase. When the enzyme is partially inactivated by preincubation with hydroxybutynoate for various times, both the hydroxy acid and amino acid oxidase activities are lost at the same rate (Figure 3), eventually producing total loss of catalytic activity. When [1-^{14}C]-2-hydroxy-3-butynoate (35 Ci/mol) was allowed to react with and completely inactivate the enzyme, the inactivated enzyme is found to contain radioactivity after Sephadex G-200 gel filtration (Figure 4). In this experiment 0.17 mg of protein contained 132,000 dpm of ^{14}C radioactivity. Since the hydroxybutynoate used was 35 Ci/mole, 0.17 mg of protein contained 1699 pmol of inactivator. This gives a value of 1 mol of inactivator/100,000g of enzyme. When this value is averaged with the results of four similar experiments, an average value of 1 mol of inactivator/102,000 g of protein was obtained. Again a ratio of flavine to subunits of 1:2 is obtained. Treatment of the inactive, radioactive enzyme with cold trichloroacetic acid followed by centrifugation to remove the denatured protein releases 98% of the radioactivity into the supernatant. These conditions totally remove all FMN from the enzyme. The radioactivity in the supernatant could be completely adsorbed on charcoal, consistent with an adduct between coenzyme and radioactive inactivator. Furthermore, the spectrum of the coenzyme released into the supernatant showed no oxidized flavine absorbance, suggesting a coenzyme modification similar to our earlier findings with a bacterial L-lactate oxidase (Walsh et al., 1972a).

Discussion

Blanchard et al. (1945) were the first to report a mammalian L-amino acid oxidase, EC 1.4.3.2, purifying the enzyme from rat kidney to perhaps 70% homogeneity and establishing its flavoprotein nature. Subsequently they found that the enzyme was even more efficient at the oxidation of L-α-hydroxy acids (Blanchard et al., 1946), and the enzyme has generally been referred to as an L-α-hydroxy acid oxidase. Nakano and colleagues subsequently crystallized the rat kidney enzyme from the mitochondrial (Nakano and Danowsi, 1966) and the soluble fractions (Nakano et al., 1967) and showed that they were physically and immunochemically identical. This identity has been questioned by Domenech et al. (1973) who found that the mitochondrial

and soluble enzymes were electrophoretically distinct and could be separated on DEAE columns. Indeed it has been shown that there is more than one α-hydroxy acid oxidase in the soluble (peroxisomal) fraction of rat kidney cells (McGroarty et al., 1974), a finding confirmed in this paper in that the soluble fraction yields three bands on acrylamide gel electrophoresis, all of which show oxidase activity. We have investigated only the major isoenzyme in the soluble fraction which appears to be identical with the single isoenzyme found in the mitochondrial fraction.

There is some question in the literature about the molecular weight of the enzyme. Nakano and Danowski (1966) first calculated a molecular weight of 89,000 but later reported a correction (Nakano et al., 1968) indicating a value of 314,000 by sedimentation equilibrium, recalculated from their earlier data. They did not directly measure the size of any subunits of the enzyme, although they determined an approximate value of one FMN/50,000 g of protein as a minimum value based on the flavine content. We have determined the subunit size to be 47,500 daltons. No evidence for molecular weight heterogeneity among the subunits was found. Since the molecular weight of the active enzyme was found to be 190,000 by Sephadex G-200 filtration, the enzyme appears to be a tetramer of identical molecular weight subunits. A molecular weight of 250,000 found by sedimentation equilibrium might suggest a pentameric structure, but a tetramer was established by the results of cross-linking experiments with dimethyl suberimidate. When the enzyme was incubated for 7 hr with a large excess of dimethyl suberimidate and then run on sodium dodecyl sulfate acrylamide gels, bands for the monomer, the dimer, and especially the tetramer were visible and gave a linear semilogarithmic plot of molecular weight vs. relative mobility on the gels (Lad and Hammes, 1974). The reason for the discrepancy of the Sephadex gel filtration and the sedimentation equilibrium data remains unknown.

In contrast to the results of Nakano and Danowski (1966) that there is one FMN/50,000 g of enzyme, we have consistently found one FMN/100,000 g of enzyme. This value was obtained both by the extinction coefficient of FMN released from fully active lyophilized samples of the enzyme and by the stoichiometry of covalent labeling of the coenzyme by radioactive 2-hydroxy-3-butynoate. Multimeric flavoenzymes reported in the literature generally exhibit one flavine per catalytic subunit. Our observation of one FMN/two subunits in L-α-hydroxy acid oxidase might be attributed to loss of half of the flavine coenzymes during isolation. This possibility cannot be unequivocally excluded despite our efforts to follow published procedures reported to yield one FMN/50,000 g of enzyme, despite our use of several different methods of homogenizing tissues, and despite inclusion of added FMN throughout purification. If half the flavine of the in vivo enzyme has been released in our preparations but not in those of Nakano et al. (1967), it might be expected that the enzymatic activity would be less, perhaps half, for the enzyme with one FMN/two subunits than for the enzyme with one FMN/subunit. However, the specific activity per milligram of enzyme protein of our preparations is identical with that reported by Nakano and Danowski (1966) suggesting the enzyme we isolated is fully competent, at least in comparison with that previously reported. It is possible that the preparations of Nakano and Danowski (1966) and Nakano et al. (1967) were one of the other isozymes we have observed in the soluble fraction and that contains one FMN/subunit. This could be resolved if enough of the minor isozymes can be recovered as pure proteins to determine FMN content.

The possibility of a very selective and reproducible proteolysis which releases some, but not all, of the flavine coenzymes cannot be completely excluded but several lines of evidence appear to argue this might not have happened. While loss of flavine coenzymes during purification of flavoenzymes has been documented (e.g., pig kidney D-amino acid oxidase), the two FMN molecules present in our preparations of rat kidney L-α-hydroxy acid oxidase are extremely tightly bound (i.e., stable to dialysis against 2 *M* KBr) and all our attempts to prepare functional apoenzyme have failed. Thus, if flavine coenzymes have been lost in our preparations during isolation, there must be an extreme functional dissimilarity in the ease of flavine release and probably in the environment of the flavines. Urea gels indicate that the isolated active tetramer is composed of two types of subunits which differ in charge but any proteolysis which might release part of the flavine does not appear to induce great dissimilarity in the size of the subunits of the enzyme, for they all appear identical on sodium dodecyl sulfate gel electrophoresis. Furthermore, De Sa (1972) has recently reported the first observation of a flavoenzyme with one flavine/two subunits from the enzyme putrescine oxidase from *Mycobacterium rubens*. Homogeneous preparations of this enzyme were found to contain one FAD/100,000 g of enzyme of identical molecular weight subunits of 50,000. This is closely analogous to our present finding. The same strictures raised above for loss of half the coenzyme might obtain in this instance as well. Nonetheless, it may be appropriate to begin considering the possibility that some flavoenzymes might be composed of a greater number of polypeptide chains than flavine coenzymes. The constitution of the active sites of these enzymes will certainly prove to be an intriguing question.

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Purification and Characterization of a Major Endonuclease from Rat Liver Nuclei†

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ABSTRACT: A major endonuclease has been purified approximately 800-fold from rat liver nuclei using poly(A) as substrate. The enzyme had a molecular weight of about 50,000, and active fractions were obtained which contained no nucleic acid. Enzymatic activity was optimal between pH 6 and 7 and was totally dependent on the presence of a divalent cation. The reaction was inhibited by high ionic strength, polydextran sulfate, heparin, and sodium pyrophosphate. The purified enzyme readily hydrolyzed poly(A), poly(U), poly(C), and denatured DNA, whereas poly(G) was not degraded, and transfer RNA, ribosomal RNA, and native DNA were hydrolyzed only at relatively slow rates. These data suggest that the enzyme may be specific for single-stranded polynucleotides. The purified enzyme was essentially devoid of exonuclease activity, and the products of exhaustive endonuclease digestion of poly(A) were small oligonucleotides terminated with a 5′-phosphoryl group. Evidence was obtained that this endonuclease is localized in the nucleoplasm. Possible functions for this activity are discussed.

It is now well established that the different polyribonucleotides of animal cells are synthesized in the nucleus as larger precursor molecules which are subsequently cleaved to generate the RNA molecules of the cytoplasm and fragments which are degraded in the nucleus (Weinberg, 1973; Darnell et al., 1973). However, very little is presently known about the nucleases which may be involved in the processing of these precursor molecules or in the breakdown of the discarded segments. Several reports have appeared previously describing the existence of nuclear endonucleases. Heppel described an endonuclease from pig liver nuclei which to date has been used mainly for the preparation of oligoribonucleotides (Heppel, 1966; Razzell, 1967). During purification of a nuclear exoribonuclease, Sporn also demonstrated the existence of a nuclear endonuclease which was not characterized further (Lazarus and Sporn, 1967; Sporn et al., 1969). More recently, nucleolar endonucleases from HeLa cells and L cells have been identified which cleave the precursor ribosomal RNA associated with preribosomal particles to the size of mature ribosomal RNA (Kwan et al., 1972; Mirault and Scherrer, 1972; Winicov and Perry, 1974). However, these nucleolar endonucleases from Novikoff hepatoma cells, HeLa cells, L cells, and rat liver show less specificity with naked 45S preribosomal RNA as substrate (Boctor et al., 1974; Kwan et al., 1972; Prestakyo et al., 1972; Winicov and Perry, 1974). The cleavage of heterogeneous nuclear RNA by an endonuclease present in 30S ribonucleoprotein particles of rat liver nuclei has also been reported (Niessing and Sekeris, 1970). Another endonuclease which may be specific for double-stranded regions of RNA has been identified in HeLa cell nuclei (Birge and Schlessinger, 1974). In order to gain further insight into the processing of RNA and the functions of nuclear nucleases, we have purified and characterized what appears to be a major endonuclease of rat liver nuclei.

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