Purification and Characterization of L-Gulonolactone Oxidase from Chicken Kidney Microsomes†

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ABSTRACT: L-Gulonolactone oxidase was purified from chicken kidney to homogeneity by a five-step method with a recovery of ca. 30%. The molecular weight of the purified enzyme under nondenaturing conditions was estimated to be 400 000 by gel filtration on Sepharose CL-6B, while that of the dissociated enzyme was 50 000 as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The latter value was comparable to the minimum molecular weight based on the content of the enzyme-bound flavin. The absorption spectrum of the enzyme showed maxima at 454, 352, and 278 nm. The spectrum in the visible and near-UV range was changed to that of the reduced form by the addition of a substrate, L-gulono-γ-lactone, under anaerobic conditions, and the reduced form of the enzyme was returned to the oxidized form by aeration. A peptide containing covalently bound flavin was isolated by tryptic–chymotryptic digestion of the enzyme and was shown to release 5′-AMP by the treatment with nucleotide pyrophosphatase, indicating that the flavin moiety is FAD. The structure of (aminoacyl)flavin derived from the peptide was identified to be 8α-(N^1-histidyl)riboflavin by high-voltage paper electrophoresis. In addition to L-gulono-γ-lactone, this enzyme attacked a number of hexonic acid lactones whose hydroxyl group on C(2) has the same configuration as that of L-gulono-γ-lactone. Stoichiometry of the enzyme reaction was determined as L-gulono-γ-lactone + O_2 → L-ascorbic acid + H_2O_2. With L-gulono-γ-lactone as the substrate, the transient appearance of an intermediate, which is considered to be 2-oxo-L-gulono-γ-lactone, was demonstrated by spectrophotometric tracing of the formation of L-ascorbic acid. The values of K_m and molecular activity obtained at low concentrations of substrate were 7 μM and 139 min^{-1}.

L-Gulonolactone oxidase [L-gulono-γ-lactone:oxygen 2-oxidoreductase, EC 1.1.3.8] is an enzyme that catalyzes the last step of L-ascorbic acid biosynthesis in animals capable of synthesizing this vitamin. Chatterjee (1973) reported that the vertebrates phylogenetically higher than fish possess this enzyme. Interestingly, it appears that its localization shifted from the kidney to the liver during evolution: mammals and highly evolved birds possess the enzyme in the liver, whereas primitive birds, reptiles, and amphibia possess it in the kidney.

Due to the low content of the enzyme in tissues as well as its localization in particulates, isolation of homogeneous enzyme has been difficult, and most of the current knowledge of the properties of L-gulonolactone oxidase is that obtained by use of crude enzyme samples. Nakagawa et al. (1975) attempted the purification from rat liver, but their preparation was not homogeneous. Although nearly homogeneous enzyme was obtained, more recently, from rat and goat livers (Nishikimi et al., 1976), the amount of the enzyme obtainable was limited. In the present study, we developed a purification procedure to obtain a large quantity of homogeneous enzyme from chicken kidney and examined its characteristics. An interesting feature of L-gulonolactone oxidase is that it contains a covalently bound flavin, as revealed with rat enzyme (Kenney et al., 1976a), and therefore, the structure of the flavin of chicken enzyme was investigated in detail. Since important properties, such as the stoichiometry of the reaction catalyzed by this enzyme and substrate specificity, have not been studied with a pure enzyme preparation, these points were also elucidated. During the course of our study, Sato & Grahn (1981) reported the purification of the enzyme from the same source. However, their main object was to examine the synthesis of L-ascorbic acid in guinea pigs after administration of the enzyme, and properties of the enzyme were not studied extensively.

Materials and Methods

Materials. Histidylflavin derivatives that were synthesized according to Edmondson et al. (1976) were donated by Dr. Nobuko Ohishi, University of Nagoya. D-Mannono-, D-glucono-, D-allono-, D-altrono-, L-idono-, and L-tarono-γ-lactone were generous gifts from Dr. Michio Matsui, Kyoritsu Pharmaceutical College, Tokyo. Other sugar–acid lactones were obtained as described previously (Nishikimi et al., 1978). Nucleotide pyrophosphatase was obtained from Sigma Chemical Co., St. Louis, MO, and the poly(ethylenimine)–cellulose plate was from Merck, AG, Darmstadt, West Germany. L-Gulonic acid was prepared as reported by Isherwood et al. (1960). All other chemicals were of analytical grade.

Enzyme Assay. The activity of L-gulonolactone oxidase was measured by the method described previously (Nishikimi et al., 1976) with some modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 50 mM sodium citrate, 1 mM dithiothreitol, 2.5 mM L-gulono-γ-lactone, and enzyme. The mixture (1.0 mL) was incubated for 15 min at 37 °C, and the reaction was stopped by the addition of trichloroacetic acid to 5%. After the precipitate that formed was removed by centrifugation, the amount of L-ascorbic acid in the supernatant was determined by the method of Zannoni et al. (1974). Since L-ascorbic acid autoxidized during the incubation, the obtained value was corrected for the autoxidation of L-ascorbic acid added to the reaction mixture without substrate. One unit of the enzyme is defined as the quantity that catalyzes the formation of 1 μmol of L-ascorbic acid/min under the specified conditions. In some experiments, the enzyme activity was measured polarographically at 37 °C by following the oxygen consumption with a YSI Model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH). So that the L-gulonolactone oxidase reaction in the initial period could be followed, the formation of L-ascorbic acid was determined spectrophotometrically by using a Union Giken stopped-flow

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spectrophotometer (Union Giken, Osaka, Japan). The rate of the reaction was calculated by using a molar extinction coefficient of 15 300 M^{-1} cm^{-1} at 265 nm (Carpēni, 1938).

Purification of L-*Gulonolactone Oxidase from Chicken Kidney.* All steps of the following purification procedure were carried out at 0–4 °C. Chicken kidneys frozen and stored overnight after excision were obtained from a poultry farm. They were homogenized in 4 volumes of 0.25 M sucrose. The homogenate was centrifuged for 15 min at 10000*g*, and then the supernatant was centrifuged for 1 h at 100000*g*. The microsomes were suspended in 1.15% KCl containing 10 mM EDTA (pH 7.4) and stored at −20 °C until use.

The microsomes (12–13 g of protein) were washed with 1.15% KCl by centrifugation and were digested with 0.03% trypsin at a protein concentration of 10 mg/mL in 20 mM Tris–acetate buffer (pH 8.0) containing 1 mM EDTA. After the mixture was allowed to stand overnight with stirring, the suspension was centrifuged for 1.5 h at 100000*g*. The digested microsomes were washed with the above Tris–acetate buffer by centrifugation. The resultant precipitate was suspended to a protein concentration of 10 mg/mL in a solution containing 4% Tween 20, 20 mM Tris–acetate buffer (pH 8.0), and 1 mM EDTA. After the suspension was stirred for 1 h, it was centrifuged for 2 h at 100000*g*. The clear supernatant was collected and dialyzed for 18 h against three changes of 10 volumes of 20 mM Tris–acetate buffer (pH 8.0) containing 10 mM KCl, 1 mM EDTA, and 0.4% Brij 35.

The dialyzed solution (1290 mL) was loaded onto a DEAE-Sephadex A-50 column (9 × 48 cm, PA 90 × 50, Amicon) previously equilibrated with the above Tris–acetate buffer used for dialysis. After the column was washed with the column volume of the Tris–acetate buffer, the proteins were eluted with 20 mM Tris–acetate buffer (pH 8.0) containing 50 mM KCl, 1 mM EDTA, and 0.4% Brij 35. The fractions with activities greater than 0.6 unit/mg of protein were combined, and the volume of the solution was brought to ca. 300 mL by ultrafiltration using an ultrafilter UK-50 (Toyo Kagaku Sangyo Co., Ltd., Osaka, Japan). The concentrated solution was dialyzed for 12 h against four changes of 5 L of 2 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.4% Brij 35.

The dialyzed solution was applied to a hydroxylapatite column (3 × 30 cm) previously equilibrated with the above phosphate buffer. After the column was washed with the same buffer, the enzyme adsorbed to the column was eluted with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.4% Brij 35. The fractions with a constant specific activity (3.43 units/mg of protein) were combined.

Purification of L-*Gulonolactone Oxidase from Rat Liver.* Rat L-gulonolactone oxidase was purified by the method reported previously (Nishikimi et al., 1976), except that in DEAE-Sephadex A-50 chromatography, a linear gradient elution was replaced with a stepwise elution, which gave a better preparation as judged from the absorption spectrum (A_{278}/A_{454} = 12.3).

Protein Determination. Protein concentration was determined by the microbiuret method (Itzhaki & Gill, 1964) using bovine serum albumin as the standard.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of 0.1% Tween 20 was carried out as described by Dewald et al. (1974), except that samples were placed on the top of separating gels without stacking gels. The activity of L-gulonolactone oxidase was stained on gels by the method described previously (Nishikimi et al., 1976). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on 7.5% gels as reported previously (Nishikimi et al., 1977). The molecular weight markers used were cytochrome *c*, chymotrypsinogen, aldolase, catalase, and bovine serum albumin. Scanning of polyacrylamide gels at 540 nm was carried out by using a Gilford 250 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH).

Sepharose CL-6B Gel Chromatography. A 0.5-mL sample of the purified enzyme (250 μg) or each of the standard protein markers (4 mg) was applied to a Sepharose CL-6B column (1.5 × 100 cm) that had previously been equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 M KCl and 1 mM EDTA. The proteins were eluted with the same buffer at a flow rate of 7.2 mL/h at 4 °C. The marker proteins used were ovalbumin, aldolase, catalase, and ferritin. The elution positions of these proteins were determined by the absorbance at 280 nm, while that of L-gulonolactone oxidase was determined by its activity.

Purification of Flavin Peptide. The following purification was carried out in the dark. L-Gulonolactone oxidase (ca. 40 mg) was precipitated by adding 9 volumes of cold acetone, and the pellet was dissolved in 2 mL of 0.1 M Tris-HCl buffer (pH 8.0) containing 8 M urea. After the mixture was allowed to stand for 2 h, the clear protein solution was diluted with 3 volumes of 0.1 M Tris-HCl buffer (pH 8.0). The protein was digested by chymotrypsin (10 μg/mg of protein) for 4 h at 38 °C and subsequently by trypsin (0.25 mg/mg of protein) for 4 h at 38 °C. After being cooled in an ice bath, the solution was mixed with 0.1 volume of 55% trichloroacetic acid, and the tryptic–chymotryptic peptide was separated from acid-insoluble materials by centrifugation at 4 °C. The peptide solution was applied to a Florisil column (0.6 × 10 cm) previously equilibrated with 5% acetic acid. The column was washed with 5% acetic acid and then with distilled water. The flavin peptide adsorbed to the column was eluted with 5% pyridine. The yellowish fraction was collected and lyophilized. The flavin peptide was dissolved in a minimum volume of 5% formic acid, and the resultant solution was chromatographed on a phosphocellulose column (1 × 5 cm) previously equilibrated with the same solvent. After the column was washed with 5% formic acid, the flavin peptide was eluted with pyridinium formate buffer (pyridine–formic acid–water, 1:5:95 v/v/v). Two distinct peaks of flavin peptide were detected in the elution pattern. The major peak of the flavin peptide was pooled and lyophilized. For the analysis of the flavin, the flavin peptide was incubated with nucleotide pyrophosphatase as described by Kenney et al. (1976b), and identification of 5′-AMP was carried out by poly(ethylenimine)–cellulose thin-layer chromatography according to Randerath & Randerath (1967).

High-Voltage Paper Electrophoresis of (Aminoacyl)flavin. The (aminoacyl)flavin was obtained from the flavin peptide by hydrolysis with 6 N HCl at 95 °C in vacuo for 16 h. High-voltage paper electrophoresis was performed at 40 V/cm for 2 h at pH 5.5 by using pyridinium acetate buffer (pyridine–acetic acid–water, 10:3.5:486 v/v/v).

Analytical Procedures. Absorption spectra were measured by using a Union Giken high-sensitivity spectrophotometer, SM-401 (Union Giken, Osaka, Japan). For measurement of the absorption spectrum of the reduced form of the enzyme, L-gulono-γ-lactone (560 μM in the final concentration) was added to a 2.5-mL solution of the enzyme (5.9 μM in terms of flavin) in a Thunberg-type cuvette under anaerobic conditions. Corrected fluorescence spectra were measured in a corrected recording spectrofluorophotometer, RF-502 (Shimadzu, Kyoto, Japan).

Table I: Purification of L-Gulonolactone Oxidase from Chicken Kidney

	total protein (mg)	total act.[a] (units)	sp act. (units/mg of protein) $\times 10^3$	yield (%)
microsomes	12 900	183	14.2	100
tryptic digestion	10 400	163	15.7	89.1
solubilization with Tween 20	3 590	126	35.1	68.9
DEAE-Sephadex A-50	132	94.4	715	51.6
hydroxylapatite	15.6	53.5	3430	29.2

[a] Enzyme activity was determined as described under Materials and Methods and expressed as units (micromoles of L-ascorbic acid formed per minutes).

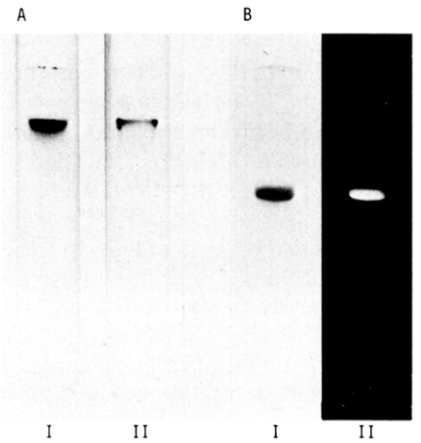

FIGURE 1: Electrophoretograms of chicken microsomal L-gulonolactone oxidase. (A) The purified enzyme (10 μg) was subjected to electrophoresis in the presence of 0.1% Tween 20 at 4 °C. Gels were stained with Coomassie brilliant blue (I) and by enzymatic activity (II). (B) The purified enzyme (10 μg) was subjected to electrophoresis in the presence of 0.1% sodium dodecyl sulfate. A gel was stained with Coomassie brilliant blue (I), and the fluorescence band was made visible under UV illumination (λ = 365 nm) after soaking a gel in 7% acetic acid (II).

Results

Purification of L-*Gulonolactone Oxidase.* A summary of the purification of L-gulonolactone oxidase from chicken kidney microsomes is shown in Table I. The purification was, in principle, based on that for rat enzyme (Nishikimi et al., 1976). Tween 20 was previously used at a concentration of 1.5% to solubilize the enzyme from rat liver microsomes. However, the same concentration of the detergent was not enough to solubilize chicken enzyme maximally. Accordingly, the concentration of Tween 20 was increased to 4%. The specific activity of this preparation was increased ca. 20-fold by DEAE-Sephadex A-50 column chromatography, and a homogeneous enzyme preparation was obtained by column chromatography using hydroxylapatite. By the present procedure, L-gulonolactone oxidase was purified 240-fold from chicken kidney microsomes with a final yield of 29%.

Polyacrylamide gel electrophoresis of the purified enzyme in the presence of 0.1% Tween 20 displayed only one protein band (Figure 1A, I). Corresponding to the protein band, L-gulonolactone oxidase activity was stained on the gel by reduction of nitro blue tetrazolium in the presence of the substrate and phenazine methosulfate (Figure 1A, II). This finding indicates that the purified preparation contains a single protein with enzyme activity. The purified enzyme migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1B, I). At the position corresponding to the enzyme, a yellow fluorescent band was observed under UV illumination when a gel was soaked in 7% acetic acid after electrophoresis (Figure 1B, II). Since the treatment of the enzyme with sodium dodecyl sulfate plus 2-mercaptoethanol in the electrophoresis procedure should release noncovalently bound substances, this result suggests that the fluorescent substance is covalently bound to the protein.

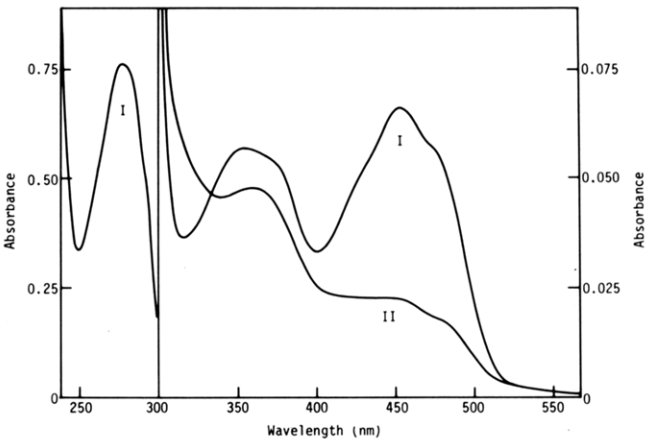

FIGURE 2: Absorption spectra of the oxidized and reduced forms of L-gulonolactone oxidase. (I) The solution of the enzyme (5.9 μM in terms of flavin) in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.4% Brij 35 in a Thunberg-type cuvette. (II) After the solution was evacuated and flushed with argon gas several times, L-gulono-γ-lactone (560 μM in the final concentration) was added anaerobically at 20 °C.

Spectral Properties. The purified enzyme showed absorption maxima at 454, 352, and 278 nm, and shoulders at 478, 426, and 292 nm (Figure 2, curve I). It is obvious from this spectral feature that the enzyme contains flavin. The ratio of A_{278} to A_{454} was 12.1. When the substrate, L-gulono-γ-lactone, was added to a solution of the enzyme under anaerobic conditions, the absorbance decreased throughout the range from 340 to 520 nm and an absorption maximum appeared at 359 nm (Figure 2, curve II). This spectrum was changed to that of the oxidized form upon aeration.

It is noted that in the absorption spectrum of the oxidized form, the second absorption maximum was hypsochromically shifted toward 352 nm as compared with that of FMN or FAD. The fluorescence intensity of the enzyme is ca. 5% of that of free FAD in 50 mM potassium phosphate buffer (pH 7.0), indicating that the fluorescence of the enzyme-bound flavin is quenched to a great extent.

Identification of the Covalently Bound Flavin of Chicken L-*Gulonolactone Oxidase.* The absorption spectrum of the tryptic–chymotryptic flavin peptide of the enzyme is shown in Figure 3. The second absorption maximum was also found to be markedly shifted to 354 nm at pH 7.3 (Figure 3, curve I) as compared with the maximum of normal flavin (370 nm), indicating that the flavin is substituted at the 8α position (Singer & Kenney, 1974). When the pH was lowered 3.4, the second absorption was further shifted hypsochromically by 5 nm (Figure 3, curve II). In accord with these changes of absorption spectrum, a similar shift of the second maximum was observed in the fluorescence excitation spectra (data not shown), and the fluorescence intensity at pH 3.4 was far higher than that at neutral pH (Figure 4, curve I). These observations suggest that histidine is the substituted amino acid in view of the properties of histidylflavin (Singer & Kenny, 1974). When the flavin peptide was treated with nucleotide pyrophosphatase, 5′-AMP was released from the flavin peptide, as demonstrated by thin-layer chromatography on poly(ethylenimine)–cellulose

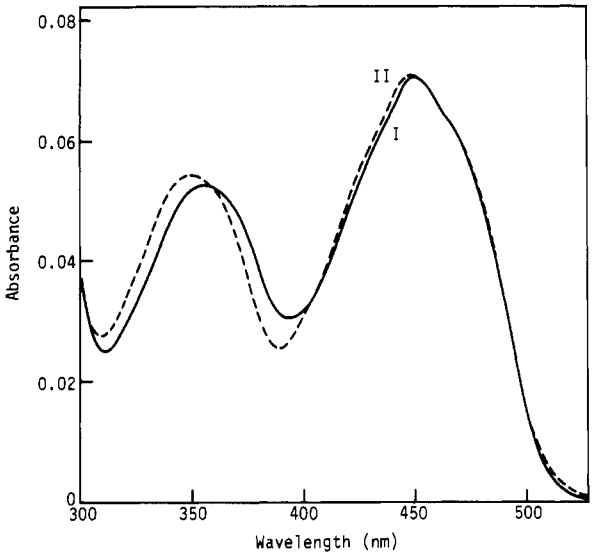

FIGURE 3: Absorption spectrum of typtic–chymotryptic flavin peptide from chicken microsomal L-gulonolactone oxidase. The flavin peptide was dissolved in 10 mM sodium citrate buffer (pH 5.0), and the pH was adjusted by the addition of 3 N NaOH or 3 N HCl to 7.3 (I) or 3.4 (II).

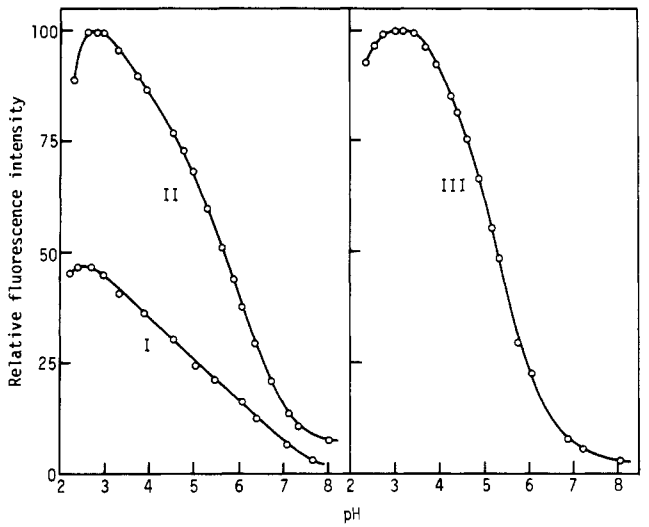

FIGURE 4: pH dependence of fluorescence intensity of flavin peptide and (aminoacyl)flavin obtained from chicken microsomal L-gulonolactone oxidase. The fluorescence intensity was measured at 535 nm with excitation at 450 nm. The pH was adjusted by the addition of 3 N HCl or 3 N NaOH. (I) Flavin peptide before treatment with nucleotide pyrophosphatase, (II) flavin peptide after treatment with nucleotide pyrophosphatase, and (III) (aminoacyl)flavin obtained after acid hydrolysis of the flavin peptide.

(data not shown). In addition, the fluorescence intensity of flavin peptide increased nearly twice at pH 3.4 after the nucleotide pyrophosphatase treatment (Figure 4, curves I and II). These observations clearly demonstrate that the flavin covalently bound to the apoprotein of chicken enzyme is at the dinucleotide level. The titration of the fluorescence intensity gave a pK_a value of 5.7 after treatment with nucleotide pyrophosphatase (Figure 4, curve II). This value is very similar to that of the flavin-peptides possessing the N(1) isomer of histidylflavin, like thiamin dehydrogenase (Kenney et al., 1974a), β-cyclopiazonate oxidocyclase (Kenney et al., 1974b), L-gulonolactone oxidase from rat liver (Kenney et al., 1976a), L-galactonolactone oxidase (Kenney et al., 1979a), and cholesterol oxidase (Kenney et al., 1979b). In contrast, the pK_a values of the flavin peptides containing the N(3) isomer of histidylflavin are nearly 1 pH unit lower (Singer & Kenney, 1974). Further confirmation of the N(1) isomer was made by comparing the properties of the (aminoacyl)flavin derived from acid hydrolysis (6 N HCl, 95 °C) of the flavin peptide with those of authentic N^1- and N^3-histidylriboflavins. The (aminoacyl)flavin obtained from the flavin peptide was separated into two components on high-voltage paper electrophoresis, and the mobility of one component (major) coincided with 8α-(N^1-histidyl)-2′,5′-anhydroriboflavin, while that of the other component (minor) coincided with 8α-(N^1-histidyl)-riboflavin (Table II). Edmondson (1977) reported that the 2′,5′-anhydro derivative is the predominant form when the flavin peptide is hydrolyzed with acid. Accordingly, the (aminoacyl)flavin primarily derived from the enzyme was assigned to 8α-(N^1-histidyl)riboflavin. This assignment was further supported by the findings that the minor component showed the pK_a value of 5.25 (Figure 4, curve III) and that it was reduced by $NaBH_4$ (Table II), since these properties distinguish the N(1) linkage from N(3) linkage (Edmondson et al., 1976). These results lead to the conclusion that the flavin of chicken L-gulonolactone oxidase is 8α-(N^1-histidyl)-FAD.

Table II: Comparison of Properties of (Aminoacyl)flavin from Chicken L-Gulonolactone Oxidase with Those of Authentic 8α-Histidylflavin Derivatives

sample	electrophoretic mobility (FMN = +1)	pK_a of imidazole	reduction by $NaBH_4$
(aminoacyl)flavin from the enzyme	−0.99[a]	5.25	yes
	−0.89[b]	5.0	yes
8α-(N^1-histidyl)riboflavin	−0.99	5.2[c]	yes
8α-(N^1-histidyl)-2′,5′-anhydroriboflavin	−0.89	5.0[c]	yes
8α-(N^3-histidyl)riboflavin	−0.64	4.7[c]	no
8α-(N^3-histidyl)-2′,5′-anhydroriboflavin	−0.56	4.5[c]	no

[a] Minor component. [b] Major component. [c] Data of Edmondson et al. (1976).

Determination of Molecular Weight. The molecular weight of the enzyme under nondenaturing conditions was ca. 400 000, as determined by gel filtration on Sepharose CL-6B. The molecular weight of the dissociated enzyme was estimated to be 50 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. If the same molar extinction coefficient is assumed for the enzyme-bound flavin as that of FAD (ϵ_{450} = 11 300) (Whitby, 1953), the quantity of protein per mole of flavin was calculated to be 51 000 g. It is concluded, therefore, that L-gulonolactone oxidase contains 1 mol of FAD/mol of monomeric form of enzyme and that the solubilized enzyme exists as an aggregate.

pH Optimum. The activity of the enzyme increased with an increase in pH to 7.5 when assayed in phosphate–citrate buffer. When Tris–citrate buffer was used, the activity was maximum at about pH 8. The activity appeared to depend on the kind of buffer, and the activity at pH 7.0 in phosphate–citrate buffer was 1.6 times higher than that in Tris–citrate buffer. Since L-ascorbic acid is unstable at alkaline pH, all of the following experiments were carried out at pH 7.0 in phosphate–citrate buffer.

Substrate Specificity. The substrate specificity of the enzyme is summarized in Table III in comparison with that of rat enzyme. Among the hexonic acid γ-lactones tested, L-gulono-, L-galactono-, D-manno-, and D-altrono-γ-lactone were oxidized by the enzyme, while none of the lactones with the C(2) hydroxyl group on the opposite side to that of the above lactones was attacked. This indicates that the enzyme has a

Table III: Substrate Specificity of L-Gulonolactone Oxidase Obtained from Chicken and Rat[a]

species	configuration of groups on C(2) and C(3)				configuration of groups on C(5)
	L-gulono-	L-idono-	L-talono-	L-galactono-	
chicken	100	<3	<3	90	
rat	100	<3	<3	70	
	D-mannono-	D-glucono-	D-allono-	D-altrono-	
chicken	64	<3	<3	16	
rat	61	<3	<3	15	

[a] The relative activities indicated are expressed as the percent of the activity with L-gulono-γ-lactone for each enzyme. The activity with various hexonic acid lactones was measured as described under Materials and Methods for L-gulono-γ-lactone at 37 °C.

configurational specificity for the hydroxyl group at C(2). L-Gulonic acid did not serve as the substrate, suggesting that the lactone structure is essential. Since D-gulono-γ-lactone was not active, it appears that the enantiomers of the substrates do not fit the active site of the enzyme. D-Glucurono-γ-lactone was also not active.

Time Course of L-*Ascorbic Acid Formation.* The formation of L-ascorbic acid due to the enzymatic oxidation of substrate can be monitored spectrophotometrically by measuring the absorbance change at 265 nm where the product, L-ascorbic acid, absorbs the light maximally. Curve I in Figure 5 shows the time course of L-ascorbic acid formation with L-gulono-γ-lactone as the substrate. It is noted that a linear increase in absorbance occurred after a short but definite lag period. When the reaction was carried out at 20 °C, the lag period became marked (data not shown). On the other hand, when oxygen consumption was followed polarographically, such a lag was not observed in an experiment under the same condition (Figure 5, curve II). Therefore, the occurrence of the lag in the absorbance change in the reaction may be interpreted to mean that there appears an intermediary product before the formation of L-ascorbic acid.

Stoichiometry of Reaction. Since there occurred a lag in the time course of L-ascorbic acid formation by this enzyme, stoichiometry of the reaction was examined at the end of the enzymatic reaction. When 0.15, 0.30, and 0.45 μmol of L-gulono-γ-lactone were added to 3-mL solutions containing a catalytic amount of the enzyme, the amounts of L-ascorbic acid formed were 0.15, 0.295, and 0.43 μmol, respectively, and the amounts of oxygen consumed were 0.15, 0.297, and 0.449 μmol, respectively. Thus it is clear that 1 mol of L-ascorbic acid is formed per mol of L-gulono-γ-lactone oxidized when 1 mol of oxygen is consumed. In another experiment, when catalase was added to the reaction mixture after the reaction proceeded to a certain extent, nearly half of the amount of O_2 consumed was regenerated, and the velocity of the oxygen consumption was reduced to about half (data not shown). It is clear from these results that hydrogen peroxide is produced

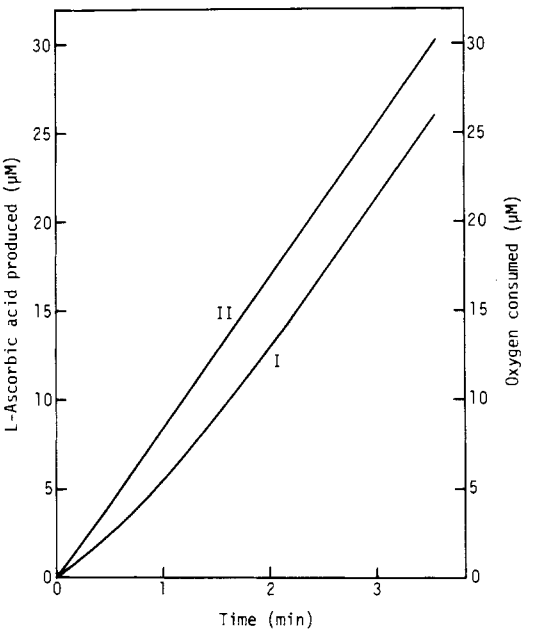

FIGURE 5: Time course of L-ascorbic acid formation by enzymatic oxidation of L-gulono-γ-lactone by chicken microsomal L-gulonolactone oxidase. (I) A solution of L-gulonolactone oxidase (100 nM in terms of flavin) was mixed with an equal volume of a solution of L-gulono-γ-lactone (10 mM) in a stopped flow apparatus. The reaction was carried out at 37 °C in 50 mM potassium phosphate buffer (pH 7.0) containing 50 mM sodium citrate in a 10-mm cell, and the absorbance change at 265 nm was followed. (II) In a parallel experiment, the reaction was carried out under the same conditions and followed by measuring oxygen consumption.

from O_2. Therefore, the stoichiometry of the enzymatic oxidation of L-gulono-γ-lactone can be expressed as

$$\text{L-gulono-}\gamma\text{-lactone} + O_2 \rightarrow \text{L-ascorbic acid} + H_2O_2$$

Kinetic Parameters. A double-reciprocal plot of molecular activity vs. the concentration of L-gulono-γ-lactone showed a break as shown in Figure 6. The values of K_m and molecular

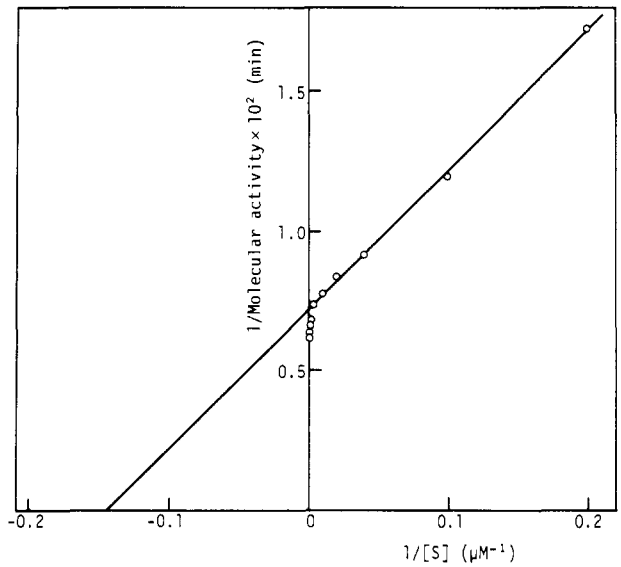

FIGURE 6: Lineweaver–Burk plot of the enzymatic oxidation of L-gulono-γ-lactone by chicken kidney L-gulonolactone oxidase. The reciprocal of the molecular activity calculated from the oxygen consumption was plotted against that of the concentration of L-gulono-γ-lactone. The experimental conditions were the same as those described in the legend for Figure 5, except for the change of the L-gulono-γ-lactone concentration (5 μM–5 mM).

activity obtained from the slope at lower concentrations of substrate were 7 μM and 139 min^{-1}, respectively. At higher concentrations of substrate (0.5–5 mM), the plot displayed an asymptotical downward curve.

Discussion

L-Gulonolactone oxidase was purified 240-fold from chicken kidney microsomes by a five-step method with relatively high yield (ca. 30%). The present method exceeds the recently reported seven-step method of Sato & Grahn (1981), by which they purified the enzyme 60-fold from the same source with a recovery of ca. 5%. Our final preparation was found to be pure from the results of polyacrylamide gel electrophoresis under both denaturing and nondenaturing conditions (Figure 1). In addition, antiserum raised in a rabbit by using this preparation has shown a single precipitin line with a crude extract from chicken kidney microsomes in the Ouchterlony test (Nishikimi et al., 1981). The data presented in this study indicate that chicken L-gulonolactone oxidase contains a flavin covalently bound to the apoprotein and that under nondenaturing conditions, it occurs as an aggregate of a monomer having a molecular weight of 50000. Similar features were previously reported for L-gulonolactone oxidase purified from rat and goat (Nishikimi et al., 1976). However, in the previous work, the quantities of protein per mole of flavin in these enzyme preparations were reported to be ca. 90000 g, and there remained a possibility that the preparations contained contaminating protein or an apoprotein of the enzyme. In the present study, the amount of protein per mole of flavin was found to be 51000 g, indicating that the molar ratio of flavin and monomeric enzyme protein is 1.

Furthermore, the purification in a large scale enables us to obtain the accurate spectral data of this enzyme. It was observed that the flavin moiety of the enzyme was reduced with substrate and oxidized upon aeration, indicating the participation of the enzyme-bound flavin in the enzymatic catalysis.

The substrate specificity of this enzyme was found to be the same as that of rat enzyme, though there were slight differences in the relative activity among various substrates (Table III). It is apparent that both enzymes show configurational specificity for the hydroxyl group on C(2). Ashwell et al. (1961) reported a similar substrate specificity for rat enzyme. However, since they used crude liver microsomal extract as the enzyme sample, it was not certain that all of these substrates were attacked by a single enzyme, L-gulonolactone oxidase. In this study, we definitely demonstrated using the homogeneous preparations of both chicken enzyme and rat enzyme that L-gulonolactone oxidase has a relatively broad specificity for the hexonic acid lactones with the same configuration of the C(2) hydroxyl group as that of L-gulono-γ-lactone.

The present results demonstrate that the stoichiometry of L-gulonolactone oxidase reaction is L-gulono-γ-lactone + O_2 → L-ascorbic acid + H_2O_2.

Spectrophotometric observation indicated that there occurs a lag before the formation of L-ascorbic acid. Since such a lag was not observed when the reaction was followed by measuring the oxygen consumption polarographically, it is considered that the dehydrogenated product, which shows little or no absorption of light at 265 nm, is initially formed and then converted to L-ascorbic acid. The site of oxidation in the substrate molecules is thought to be the hydroxyl group on C(2) (Chatterjee et al., 1959), and it is conceivable that the primary product is 2-oxo-L-gulono-γ-lactone, which gives L-ascorbic acid by spontaneous isomerization.

Kinetic study demonstrated a deviation from the Michaelis–Menten kinetics at high concentrations of the substrate. The elucidation of its mechanism awaits further investigation.

Chicken L-gulonolactone oxidase was found to possess a flavin covalently bound to the apoprotein and its structure was identified as 8α-(N^1-histidyl)-FAD. Kenney et al. (1976a) reported that the structure of rat enzyme is 8α-(N^1-histidyl)flavin and suspected the flavin to be FAD in view of the increase of fluorescence intensity upon treatment of nucleotide pyrophosphatase, though the definite evidence was lacking. In the present study, we definitely showed by observing the release of 5′-AMP that the flavin peptide of chicken enzyme contained FAD. Besides the occurrence of the covalently bound flavin, chicken enzyme has similar minimum molecular weight (50000) to that of rat enzyme, and both enzymes have analogous substrate specificity. Furthermore, the antiserum directed to the chicken enzyme shows cross-reactivity to rat enzyme (Nishikimi et al., 1981). Although these enzymes are localized in different organs, viz., chicken enzyme being in the kidney and rat enzyme being in the liver, the above-mentioned similarity between them suggests their homology.

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Effects of *S*-Adenosyl-1,8-diamino-3-thiooctane on Polyamine Metabolism†

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ABSTRACT: Exposure of mammalian cells (transformed mouse fibroblasts or rat hepatoma cells) to *S*-adenosyl-1,8-diamino-3-thiooctane produced profound changes in the intracellular polyamine content. Putrescine was increased and spermidine was decreased, consistent with the inhibition of spermidine synthase by this compound, which is a potent and specific "transition-state analogue inhibitor" of the isolated enzyme in vitro. The spermine content of the cells was increased by exposure to this drug presumably since spermine synthase was able to use a greater proportion of the available decarboxylated *S*-adenosylmethionine when spermidine synthase was inhibited. The decarboxylated *S*-adenosylmethionine content rose substantially because the activity of *S*-adenosylmethionine decarboxylase was increased in response to the decline in spermidine. These results indicate that *S*-adenosyl-1,8-diamino-3-thiooctane is taken up by mammalian cells and is an effective inhibitor of spermidine synthase in vivo and that *S*-adenosylmethionine decarboxylase is regulated by the content of spermidine, but not of spermine. The growth of SV-3T3 cells was substantially reduced in the presence of *S*-adenosyl-1,8-diamino-3-thiooctane at concentrations of 50 μM or greater. Such inhibition was reversed by the addition of spermidine but not by putrescine. When SV-3T3 cells were exposed to 5 mM α-(difluoromethyl)ornithine and 50 μM *S*-adenosyl-1,8-diamino-3-thiooctane, the content of all polyamines was reduced. Putrescine and spermidine declined by more than 90% and spermine by 80%. Such cells grew very slowly unless spermidine was added.

Polyamines are synthesized in mammalian cells from ornithine and AdoMet[1] by the actions of four key enzymes: ornithine decarboxylase, AdoMet decarboxylase, spermidine synthase, and spermine synthase. The two decarboxylases provide putrescine and decarboxylated AdoMet. Spermidine synthase catalyzes the transfer of an aminopropyl group from decarboxylated AdoMet to putrescine forming spermidine and 5′-(methylthio)adenosine. Spermine synthase transfers a second aminopropyl group from another molecule of decarboxylated AdoMet to spermidine forming spermine and 5′-(methylthio)adenosine (Tabor & Tabor, 1976; Jänne et al., 1978; Pegg & Williams-Ashman, 1981; Williams-Ashman & Pegg, 1981). The function(s) of polyamines in cellular physiology is (are) still not well understood, but evidence from experiments in which the polyamine content was depleted by exposure to inhibitors of biosynthetic enzymes or by mutations affecting these enzymes suggest that they are essential for normal growth (Tabor & Tabor, 1976; Jänne et al., 1978; Cohn et al., 1978, 1980; Morris, 1981; Tabor et al., 1981). Although mutations affecting the activity of each of the key biosynthetic enzymes have been produced in yeast (Cohn et al., 1978, 1980), such mutants are not available in mammalian cells except for CHO cell lines lacking ornithine decarboxylase (Steglich & Scheffler, 1982; Pohjanpelto et al., 1981). Studies of the roles of polyamines in mammalian cell physiology have, therefore, mainly been carried out by using inhibitors (Jänne et al., 1978; Heby & Jänne, 1981; McCann et al., 1981; Koch-Weser et al., 1981; Heby, 1981; Porter et al., 1981).

Many attempts to synthesize potent and specific inhibitors have been made, and some useful inhibitors have resulted that may have valuable pharmacological actions (McCann et al., 1981; Koch-Weser et al., 1981; Porter et al., 1981). However, all of the inhibitors presently available have disadvantages, and at present, only certain inhibitors of ornithine and AdoMet decarboxylases have effects on cellular metabolism that are reversed by addition of polyamines and thus pass a first test

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[1] Abbreviations: AdoDATO, *S*-adenosyl-1,8-diamino-3-thiooctane; AdoMet, *S*-adenosylmethionine; DFMO, α-(difluoromethyl)ornithine.

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