The Partial Purification and Properties of Hydroxylysine Kinase from Rat Liver*  

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SUMMARY

Hydroxylysine kinase (guanosine triphosphate:5-hydroxy-L-lysine O-phosphotransferase; EC 2.7.1.-) has been purified 1200-fold from rat liver and its properties partially elucidated. The enzyme catalyzes the phosphorylation of allohydroxy-L-lysine and hydroxy-L-lysine with an apparent Kₗₑ₄ ~ 6 μM, but has no activity with either of the D isomers. The maximum velocity with allohydroxy-L-lysine was 1.5 to 3 times greater than with hydroxy-L-lysine. GTP or hypoxanthine triphosphate serve as the phosphate donor. Mn²⁺ is specifically required for the reaction while both Mn²⁺ and Zn²⁺ are inhibitory. The active phosphorylating agent appears to be the MgGTP⁻⁻ complex with guanosine diphosphate as the nucleotide product. The enzyme is present in the liver or kidney, or both, of rats, mice, chickens, bovine, rabbits, and two species of primates.

LIVER. A preliminary report of some of these results has been made (11).

EXPERIMENTAL PROCEDURE

Materials—Mixed isomers of 5-hydroxy-L-[²⁴°C]lysine were synthesized (12) by the method of Lindstedt (13) and separated into the two racemates (14). These were resolved into the individual isomers according to Friede et al. (12). The specific activity was approximately 6 mCi per mmole. Hydroxy-L-lysine was isolated from gelatin by the procedure of Hamilton and Anderson (15) and found to be free of the allo-racemate by means of the amino acid analyzer. Trans 4,5 dehydro L-lysine dihydrochloride and S-aminoethyl-L-cysteine monohydrochloride were a gift of Dr. A. L. Davis, Abilene Christian College. 2-Amino-5-hydroxypipdic acid hydrochloride was prepared by the hydrolysis of the cyanohydrin intermediate formed during the synthesis of hydroxylysine (13). All other chemicals were of the purest grade available from commercial sources.

Enzyme Assays—Two basic assay systems were used in the experiments. The final concentration of the components for Assay I was 0.14 M HEPES (pH 8.0 with NaOH), 1 mM MgCl₂, 1 mM GTP, 2 mM ATP, 3 mM creatine phosphate, 10 units of creatine kinase (Worthington), 40 μM hydroxy-L-[¹⁴C]lysine with tissue extract and water to give a volume of 5 ml. The final concentration of the components for Assay II was 0.14 M HEPES, 1 mM MgCl₂, 1 mM GTP, 40 μM hydroxy-L-[¹⁴C]lysine and enzyme and water to give a volume of 5 ml. The assays were done at 37°C and initiated by the addition of enzyme. The reaction was stopped usually after 30 to 60 min by bringing the solutions to 50% ethanol, and the precipitate was removed by centrifugation at 15,000 x g. The contents of each reaction vessel were mixed with 2 ml of Dowex 1-X8 acetate (200 to 400 mesh) suspension³ and adjusted to pH 8.0 to 8.5 with NaOH. This mixture was poured on the top of a column (1.1 x 33 cm) already packed with 2 ml of the Dowex 1 suspension. The protein and some of the precipitate were collected in one tube. The column was washed further with 25 ml of water which was collected into a second tube. A third tube collected the effluent from 25 ml of 0.1 M formic acid addition.

† The abbreviations used are: HEPES, N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid.

‡ 100 ml of settled Dowex 1-X8 acetate (200 to 400 mesh) diluted to 150 ml with water.

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justed to pH 5.2 with pyridine. The total radioactivity in each tube was determined by liquid scintillation counting. Since previous experiments with pure compounds had shown that the first two tubes contained unreacted hydroxy-L-lysine and the third tube only phosphohydroxylysine, the yield of product formed could be readily calculated. Control reactions containing no enzyme were run with groups of assays and any radioactivity in the pyridine-formate eluate (usually <1.5% of the total applied counts) was subtracted from the experimental values. One unit of enzyme activity was that amount which catalyzed the formation of 1 umole of phosphohydroxylysine per min. Relative protein concentration was determined from the absorbance at 260 and 280 nm.

**RESULTS**

**Purification of Enzyme**

**Extraction—**Male rats which had been fasted overnight were killed by decapitation and the livers quickly removed and chilled. All purification procedures were performed at 0-4°C. The tissue was minced and extracted in a Potter-Elvehjem homogenizer with 2 volumes of denatured ice water containing 1 mM GSH (pH 7.4) and 10 mM EGTA (pH 7.4). The extract was centrifuged for 30 min at 33,000 x g and the activity of the supernatant fluid, called the crude fraction, evaluated with Assay I. There was no activity in the mitochondrial or microsomal fraction.

**Acid Treatment—**The crude fraction was diluted with an equal volume of the extraction buffer, and 0.3 M HCl was added with rapid stirring to pH 3.2. This pH was maintained for 3 to 5 min after which 1 M NaOH was added to bring the solution to pH 5.2. The heavy precipitate was removed by centrifugation at 15,000 x g, and the pH of the supernatant fluid adjusted to pH 7.4 and, if necessary, recentrifuged. The enzyme activity was determined with Assay II and the fraction was designated acid fraction.

**Ammonium Sulfate Fractionation—**To the acid fraction, solid ammonium sulfate was added to a final concentration of 40% saturation and stirring continued for a total period of 2 hours. The precipitate was removed by centrifugation and the supernatant fluid brought to a final concentration of ammonium sulfate of 50% saturation. The protein was pelleted by centrifugation and resuspended in 0.2 volume of extraction buffer for each volume of the acid fraction. This enzyme preparation was dialyzed for 16 hours against approximately 70 volumes of a 4 mM HEPES-0.1 M NaCl (pH 7.4) buffer containing 0.5 mM GSH and 5 mM EGTA. The activity of this ammonium sulfate fraction was determined with Assay II.

**DEAE-Sephadex—**DEAE-Sephadex A-50 was equilibrated with 4 mM HEPES-0.1 M NaCl (pH 7.4) and a column (2.5 x 40 cm) prepared. The dialyzed ammonium sulfate fraction was placed on the column and then washed with 4 mM HEPES-0.15 M NaCl (pH 7.4), 0.2 mM GSH, and 2 mM EGTA until the largest protein peak was removed (Fig. 1). The column was then washed with the same buffer as above, except with the NaCl concentration increased to 0.15 M until the enzyme was removed. The appropriate fractions were pooled, concentrated to approximately 0.2 mg of protein per ml by means of ultrafiltration through a 10,000 molecular weight exclusion membrane, and the activity was determined with Assay II.

A purification ranging from 850- to 1200-fold was achieved by these methods with recoveries between 75 to 100%. The enzyme could be stored frozen for weeks with little loss of activity. A summary of the purification from 49.5 g of fresh rat liver is given in Table I.

**Properties of Partially Purified Enzyme**

**Velocity as Function of Duration of Catalysis—**As measured with Assay I, the rate of the reaction with crude enzyme was linear only for the first hour (Fig. 2). The fact that purified phosphohydroxylysine incubated in the presence of crude liver extract, but with no nucleoside triphosphate was not degraded to hydroxylysine indicated that the product of the reaction was stable to liver phosphatases. Fig. 2 shows that following the acid treatment of the crude extract, the reaction became linear for at least 2 hours. To determine if the early limitation with crude enzyme could be due to depletion of nucleoside triphosphate, 10 μmoles of ATP with no hydroxylysine was incubated with crude and acid-treated extract. During 1 hour at 37°C, there was no release of more than 7 μmoles of inorganic phosphate in the crude extract while no detectable phosphate was released by the acid-treated enzyme. The results indicated that crude liver extracts contained ATPase which was acid labile.

**Nucleoside Triphosphate Specificity—**The enzyme had a requirement for GTP which was partially met by ITP (Table II).
**TABLE II**
Various nucleoside triphosphates as phosphate donors in hydroxylysine kinase reaction

The data were obtained with 2.9 units of the DEAE-fraction enzyme. Assay II as described in the text was used except that GTP was replaced with an equimolar concentration of another nucleoside triphosphate.

<table>
<thead>
<tr>
<th>Nucleoside triphosphate</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>2</td>
</tr>
<tr>
<td>CTP</td>
<td>6</td>
</tr>
<tr>
<td>UTP</td>
<td>6</td>
</tr>
<tr>
<td>ITP</td>
<td>90</td>
</tr>
</tbody>
</table>

Of interest, but not shown here, was the observation that ATP would allow the formation of phosphohydroxylysine without the addition of any guanosine phosphate in the crude and acid fractions. However, after dialysis or ammonium sulfate fractionation, GTP was required for activity. Additional experiments indicated that the activity with ATP was due to the nucleoside diphosphokinase-catalyzed phosphorylation of small amounts of GDP which were in the tissue extract, to form GTP. The nucleoside product of the hydroxylysine kinase reaction was GDP with only small amounts of GMP detected (Fig. 3).

**Metal Ion Specificity** Hydroxylysine kinase required the presence of a divalent cation for activity with a strong preference for Mg$^{2+}$ (Assay II). The following metal salts at 1 mM gave 13% or less of the activity found with an equal concentration of Mg$^{2+}$: HgCl$_2$, CuCl$_2$, CoCl$_2$, FeCl$_3$, CoCl$_3$, and MnCl$_2$. In addition, the inclusion of 1 mM ZnCl$_2$ or AlCl$_3$ with the same

**Fig. 2.** The formation of phosphohydroxylysine with time. Assay I was used with either 0.55 unit of crude fraction enzyme (●) or 0.45 unit of acid fraction enzyme (△).

**Fig. 3.** The nucleoside phosphate products of the hydroxylysine kinase reaction. DEAE-fraction enzyme (1.67 units) was incubated with 0.25 mM MgCl$_2$, 0.25 mM GTP, 2 μCi of [8-14C]-GTP (36 μCi per μmole), 0.14 M HEPES (pH 8.0), and water and hydroxy-L-lysine to 5 ml. The control reaction (——) contained no hydroxy-L-lysine while the experimental reaction (●) was 60 μM hydroxy-L-lysine. At the end of the 2-hour incubation period at 37°C, 3 mg each of GMP, GDP, and GTP were added and the reactions heated in boiling water for 3 min. The reaction mixtures were passed through separate Dowex 1-X8 formate (200 to 400 mesh) columns (1.2 × 25 cm) and washed with 50 ml of water. The three nucleoside phosphates were then resolved by elution with formic acid or formic acid and ammonium formate as shown.

**Fig. 4.** The effect of manganese ion on the activity of hydroxylysine kinase. Using Assay II, 1 mM MgCl$_2$ and 1.3 units of the DEAE-fraction enzyme, the relative kinase activity was measured at MnCl$_2$ concentrations from 0 to 0.4 mM.
FIG. 5. Estimation of the apparent $K_m$ and $V_{max}$ of hydroxyllysine kinase with hydroxy-L-lysine (O) or allohydroxy-L-lysine (■) as substrate. The rate of phosphorylation was determined using 1 unit of enzyme in Assay II with the appropriate isomer of hydroxyllysine as the substrate. The initial rates at each concentration were determined from the slope of a curve obtained by plotting product formation against time with observations at 10, 20, and 30 min. The velocity remained relatively constant for 1 to 2 hours (See Fig. 2).

concentration of $\text{MgCl}_2$ inhibited the kinase to 13% of the control activity. Other metal salts were not tested under these conditions. It was found that only 25 µM $\text{MnCl}_2$ in the presence of 1 mM $\text{MgCl}_2$ was necessary to inhibit the phosphorylation of hydroxy-L-lysine 50% (Fig. 4). The inclusion of 1 mM $\text{KCl}$ or $\text{LiCl}$ in Assay II neither stimulated nor inhibited the kinase. Since the buffer used in the assays was in the sodium form it was assumed that sodium did not adversely affect the kinase.

Specificity Toward Hydroxyllysine Isomers—Neither hydroxy-D-lysine nor allohydroxy-D-lysine was phosphorylated by hydroxyllysine kinase while both of the L isomers were acted upon. The apparent $K_m$ values were of 5.6 µM and 6.2 µM for hydroxy-L-lysine and allohydroxy-L-lysine, respectively (Fig. 5). However, the maximum rate for the allo-form was 1.5 to 3 times greater than for hydroxy-L-lysine (Fig. 5). The kinase reaction, as measured with Assay II using DEAE-fraction enzyme and 35 µM allohydroxy-L-[14C]lysine was inhibited by the inclusion of nonradioactive hydroxy-L-lysine (17% at 35 µM and 70% at 350 µM) indicating that one enzyme was catalyzing the phosphorylation of both substrates.

Effects of pH—Hydroxyllysine kinase exhibited a sharp optimum activity at pH 7.7 as determined with a combination of HEPES and glycine buffers in the sodium form (Fig. 6). The inhibition of the kinase in the presence of $N$-tris(hydroxymethyl)methylglycine buffer can be accounted for by the known ability of this compound to chelate $\text{Mg}^{2+}$ (16).

Effects of Concentration of $\text{MgCl}_2$ and GTP—The reaction rate of the kinase was constant over a range of saturating concentra-

FIG. 6. The effect of pH on hydroxyllysine kinase activity. Each point in the figure was determined using Assay II, 1 unit of DEAE-fraction enzyme, and a 0.14 M concentration of either $N$-tris(hydroxymethyl)methylglycine (●), HEPES (○), or glycine (□) buffer. The pH was measured half-way through the 1-hour reaction time with a single probe electrode previously equilibrated and standardized at 37°C.

Fig. 7. The effects of the GTP and $\text{Mg}^{2+}$ concentrations on hydroxyllysine kinase activity. DEAE-fraction enzyme (2.9 units) was used in Assay II. The control point (●) contained an equimolar concentration (1 mM) of total GTP and $\text{MgCl}_2$. The activity was then determined and compared to the control while holding total $\text{MgCl}_2$ constant and increasing total GTP (○) or maintaining total GTP constant and increasing the total $\text{MgCl}_2$ (□). The points on the inset were determined using Assay II and DEAE-fraction enzyme with changing levels of both total GTP and total $\text{MgCl}_2$, holding the total molar ratio at unity (▲).
Effects of various hydroxy-containing compounds on formation of phosphohydroxylysine

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relative activity</th>
<th>4 mM$^a$</th>
<th>46 mM$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>%</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>%</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td>L-Serine</td>
<td>%</td>
<td>87</td>
<td>88</td>
</tr>
<tr>
<td>L-Homoserine</td>
<td>%</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>%</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>Choline</td>
<td>%</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>2-Amino-5-hydroxyadipate (4 isomers)</td>
<td>%</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>Carnitine (2 isomers)</td>
<td>%</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>Hydroxy-L-proline</td>
<td>%</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Hydroxy-L-piperidolate</td>
<td>%</td>
<td>54</td>
<td>54</td>
</tr>
</tbody>
</table>

$^a$ Concentration of inhibitor.

analogues of this enzyme and by whole animal experiments (6). Because of the specificity toward the naturally occurring substrates, hydroxy-L-lysine and GTP, this enzyme should be designated GTP:5-hydroxy-L-lysine O-phosphotransferase (EC 2.7.1.-) with the common name of hydroxylysine kinase.

Hydroxylysine kinase appears to be the only enzyme in higher animals capable of the direct phosphorylation of a free amino acid. An enzyme with a similar function, homoserine kinase, has been studied, but seems to exist only in microorganisms and to utilize ATP. By means of the competitive inhibitor method of screening potential substrates, at 1000-fold excess molar concentration, hydroxylysine kinase was not strongly inhibited by any of the L forms of the common hydroxy-substituted amino acids except hydroxy-L-proline. Labeled hydroxyproline was not phosphorylated by this enzyme. The structural analogue, 2-amino-5-hydroxyadipate, which has been proposed as an intermediate in the hydroxyproline metabolism (17, 18) and thus might exist in the chicken (6) is inhibitory at the high concentration tested. In addition to the competitive inhibitor data, the very low $K_m$ value of 5.6 $\mu$mol for hydroxy-L-lysine indicates that the physiological function of this enzyme is to initiate the degradation of hydroxy-L-lysine. Calculations indicate that the liver of a 360-g rat is capable of metabolizing 240 $\mu$moles of hydroxy-L-lysine per day through the liver kinase under optimal conditions. This level would represent a minimum potential since the kidneys also contain substantial quantities of the kinase. These calculations are not inconsistent with whole animal experiments showing a capacity to degrade 200 $\mu$moles of hydroxy-L-lysine within a 24-hour period (6). Hydroxylysine kinase is present in the kidney and liver of several higher animals.

$^a$ The primate tissue was kindly provided by Dr. U. S. Seal, Veterans Administration Hospital, Minneapolis, Minn.

$^4$ Unpublished observation.
including chicken, rat, mouse, bovine, rabbit, and an old and a
new world monkey. The explanation for the apparent absence
of activity in human tissues requires additional investigation.

Of particular interest is the requirement of hydroxylysine
kinase for GTP or ITP. Only three other such GTP specific
kinases have been observed in higher animals, (a) succinate
thiokinase (EC 6.2.1.4) (19) of pig heart which requires Mg$^{2+}$
and will utilize ITP, (b) phosphoenolpyruvate carboxykinase
(EC 4.1.1.32) (20) of pig liver which requires Mn$^{2+}$ and will
use ITP, and (c) adenylosuccinate synthetase (EC 6.3.4.4).5
A reasonable explanation for such a specificity toward GTP has
not been offered since hydroxylysine kinase would seem to func-
tion only to remove a metabolic end product with no critical
role in energy balance.

From the data reported above a few statements concerning
the active site and mechanism of action of hydroxylysine kinase
can be made. The almost equal $K_m$ values for allohydroxy L-
lysine and hydroxy-L-lysine, the unequal maximum velocity
with the two compounds, and the inability to utilize either of the
D isomers demonstrate that the binding of the substrate to the
enzyme is controlled primarily by the configuration at the $\alpha$
carbon atom while the rate of phosphate transfer is determined
by the configuration of the hydroxyl group relative to the
$\alpha$-amino group. This was reinforced by the greater ability of
the L isomers of nonhydroxy-containing analogue compounds,
e.g. lysine, to inhibit the kinase as compared to the corresponding
D isomer. The requirement for both Mg$^{2+}$ and GTP and the
inhibitory nature of excess free GTP indicates that as with other
kinases (22), a metal ion-GTP complex is the active substrate
in the reaction. The mechanism of binding of substrates and
the nature of the metal ion and substrate analogue inhibition
will require more sophisticated kinetic analyses.

5 Adenylosuccinate synthetase of higher animals has not been
well characterized; however, the enzyme from Escherichia coli
functions only poorly with ITP and has optimal activity with
Mg$^{2+}$ rather that Mn$^{2+}$ (21).
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