Purification and Characterization of an α-Ketoisocaproate Oxygenase of Rat Liver*

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Rat liver contains a cytosolic α-ketoisocaproate oxygenase which oxidatively decarboxylates and hydroxy-ylates α-ketoisocaproate to form β-hydroxyisovalerate. This oxygenase was purified to near homogeneity. The oxygenase is unstable during purification, unless 5% monothioglycerol is added. The purified enzyme is stable in the presence of 5% monothioglycerol for 3 weeks at 4 °C and at least 10 weeks at −80 °C. The molecular weight of the α-ketoisocaproate oxygenase was determined to be 46,000 and 51,000 using denaturing and non-denaturing conditions, respectively, indicating a monomer. The α-ketoisocaproate oxygenase requires Fe**; other metal ions did not replace Fe**. Ascorbate activates the enzyme at subsaturating levels of Fe**, by regenerating Fe**. The activity is markedly affected by the type of buffer used. For example, the oxygenase activity increased 2- to 3-fold when 0.1 M maleate was used. Iron chelators, such as ADP and EDTA, are inhibitory. The ratio of decarboxylation of 1 mm α-[1-14C]ketocaprate (as measured by 14CO2 release) to decarboxylation of 1 mm α-[14C]ketomethylbutyrate is 1.0 for all purification fractions, indicating that a single enzyme catalyzes the decarboxylation of both substrates. The apparent Km and Vmax values of the α-ketoisocaproate oxygenase using optimized assay conditions are 0.32 mM and 130 nmol/min/mg of protein for α-ketoisocaproate and 1.5 mM and 247 nmol/min/mg of protein for α-ketoγ-methylisobutyrate. The principal product of the purified α-ketoisocaproate oxygenase, using α-ketoisocaproate as a substrate, is β-hydroxyisovalerate, although small amounts of a compound, which has the chromatographic properties of isovalerate, are also produced.

Wohlhueter and Harper (1) first reported the decarboxylation of α-ketoisocaproate, the α-keto analogue of leucine, by a soluble fraction from rat liver. They recognized that this activity was not “leaked” mitochondrial branched-chain α-keto acid dehydrogenase. Subsequently, Grant and Connolly (2) reported that α-ketoisocaproate, but not α-ketoisovalerate or α-keto-β-methylvalerate, is decarboxylated by cytosolic preparations from liver and kidney of mouse, rat, rabbit, guinea pig, and cow and also from chicken liver. This decarboxylase did not use CoASH or NAD+, cofactors required for the mitochondrial branched-chain α-keto acid dehydrogenase.

Cytosolic preparations from rat liver decarboxylate both α-KIC and α-keto-γ-methylisobutyrate, the α-keto acid of methionine, in the presence of O2 (3), indicating that the enzyme is an oxygenase. Partially purified preparations from rat liver convert α-KIC to β-hydroxyisovalerate. Isovalerate is not an intermediate of this reaction (3).

In order to further characterize this enzyme and to determine whether a single enzyme is responsible for the above-mentioned observations, the α-KIC oxygenase from rat liver was purified to near homogeneity. Some of the physical and kinetic properties and the purification of the α-KIC oxygenase are described herein.

EXPERIMENTAL PROCEDURES

Materials

L-[1-14C] Leucine and L-[U-14C] leucine were obtained from ICN Pharmaceuticals, Irvine, CA. L-[1-14C] Methionine was obtained from Amersham Corp.

Methods

Preparation of Radioactively Labeled α-Keto Acids—[1-14C] labeled α-keto acids were prepared from the 14C-labeled l-amino acids, and purity was determined according to the method of Rudiger et al. (4). Solutions of 14C-labeled α-keto acids were made to their final concentrations by addition of unlabeled α-keto acids (Sigma, sodium salt) and stored at −80 °C.

Assay of α-Ketoisocaproate Oxygenase Activity—Assays for determination of α-KIC oxygenase were carried out in culture tubes (1.5 × 8.5 cm). Each tube contained in a final volume of 0.4 ml 0.2 M Tris base, 0.2 M maleic acid, pH adjusted to 6.5 with NaOH, 1.0 mM FeSO4, 0.5 mM ascorbic acid, 1.0 mM dithiothreitol, 10.0 mM α-[1-14C]KIC (approximately 100 dpm/nmol), and 5-100 μl of enzyme. A stock solution containing 16 mM FeSO4, 8 mM ascorbic acid, and 16 mM dithiothreitol (cofactor mix) was prepared fresh daily and 25 μl was added/assay just before (within 20 min) enzyme addition (5). The enzyme was preincubated in the reaction mixture for 1 h at 25 °C (unless noted otherwise) with all components excluding the substrate, α-[1-14C]KIC. The α-[1-14C]KIC was added to initiate the reaction and the culture tube tightly stoppered with a serum cap. The plastic cup (Kontes) hanging from this cap had previously been filled with 0.2 ml of Hyamine (1 M methylenethiosulfonium hydroxide in methanol; Sigma). 

Incubations were for 60 min (unless noted otherwise) in a shaking water bath. The reactions were terminated by addition of 0.2 ml of 20% trichloroacetic acid and an additional hour with shaking was allowed for collection of 14CO2. The cup plus Hyamine was transferred to a scintillation vial and counted in 10 ml of scintillation fluid (6). Specific activity of α-[1-14C]KIC was determined by releasing all of the 14CO2 using ceric sulfate as described previously (7). Nonenzymatic decarboxylation of the α-KIC was determined by replacing the α-KIC oxygenase with buffer in the assay. These blank values were the same as those obtained with boiled enzyme preparations.

Purification of α-Ketoisocaprate Oxygenase—The α-KIC oxy-
Rat Liver α-Ketoisocaproate Oxygenase

This page discusses the purification and characterization of rat liver α-Ketoisocaproate Oxygenase (a-KIC oxygenase) using various chromatographic and electrophoretic methods. The enzyme was purified from rat liver homogenates using a series of chromatographic steps, including column chromatography on Sephacryl S-200, Phenyl-Sepharose CL-4B, and DEAE-cellulose. The purified enzyme was characterized for molecular weight and molecular structure.

Purification Steps:

- ammonium sulfate precipitation
- dialysis
- DEAE-cellulose column chromatography
- Phenyl-Sepharose CL-4B column chromatography
- Sephacryl S-200 column chromatography

Results and Conclusion:

The a-KIC oxygenase activity was purified to homogeneity, with a yield of 30% of the eluent. The enzyme was found to be a monomer with a molecular weight of 30,000. The activity was stable at -80 °C and was inhibited by 0.6% monothioglycerol. The enzyme was resolved using 10% acrylamide gels and stained with Coomassie blue dye. The results were compared with those of Bonner and Laskey, who observed similar properties for a-KIC oxygenase from chicken liver.
Glyceraldehyde 3-phosphate dehydrogenase, purified from rat liver, was used. For example, when 0.4 ml of the concentrated DEAE-pool (Table I) was applied to a Sephacryl S-200 column, large losses of activity occurred during purification when DEAE-cellulose, phenyl-Sepharose, or Sephacryl columns were used. For example, when 0.4 ml of the concentrated DEAE-pool (Table I) was applied to a Sephacryl S-200 column (1.3 × 56 cm), the recovery of α-KIC oxygenase was less than 10%. Elution with high concentrations of NaCl gave additional protein but did not increase the yield of α-KIC oxygenase. Pretreatment of the Sephacryl S-200 column with protein-containing fractions recovered from the DEAE-cellulose column, which did not contain α-KIC oxygenase (DEAE-side fractions), increased the recovery to 82%. Kaufman and Fisher (12) used a similar technique for purification of phenylalanine hydroxylase from rat liver.

RESULTS

Stabilization of the α-Ketoisocaproate Oxygenase—Initial attempts to purify the α-KIC oxygenase yielded very low recoveries. During these studies it was shown that inclusion of FeSO₄, ascorbate, and dithiothreitol in assays produced a 3- to 4-fold stimulation of activity (3). With the partially purified α-KIC oxygenase, optimal concentrations of FeSO₄, ascorbate, and dithiothreitol were 1.0, 0.5, and 1.0 mM, respectively. Despite the inclusion of these compounds in all assays, large losses of activity occurred during purification when DEAE-cellulose, phenyl-Sepharose, or Sephacryl columns were used. For example, when 0.4 ml of the concentrated DEAE-pool (Table I) was applied to a Sephacryl S-200 column (1.3 × 56 cm), the recovery of α-KIC oxygenase was less than 10%. Elution with high concentrations of NaCl gave additional protein but did not increase the yield of α-KIC oxygenase. Pretreatment of the Sephacryl S-200 column with protein-containing fractions recovered from the DEAE-cellulose column, which did not contain α-KIC oxygenase (DEAE-side fractions), increased the recovery to 82%. Kaufman and Fisher (12) used a similar technique for purification of phenylalanine hydroxylase from rat liver.

Despite pretreatment of phenyl-Sepharose and Sephacryl columns with DEAE-side fractions, large losses of α-KIC oxygenase activity occurred. Although the α-KIC oxygenase was quite stable at 4 °C in less pure preparations such as the concentrated DEAE-pool (9% loss of activity in 4 days), the purified preparations of α-KIC oxygenase rapidly lost activity (50% loss of activity in 4 days, data not shown). This loss was apparently not dependent on the protein concentration, since dilution of the concentrated DEAE-pool from 16.8 to 1.68 mg of protein/ml did not alter stability. The α-KIC oxygenase was stable at −80 °C for up to 20 days in all purification fractions; however, at −20 °C or room temperature activity was rapidly lost (5). The α-KIC oxygenase is more stable at 4 °C than at −80 °C or room temperature.

Use of 5% monothioglycerol (0.6 M) stabilized the α-KIC oxygenase at 4 °C for at least 6 days (Table II), but 1% monothioglycerol (0.12 M) was not effective. Dithiothreitol at 1 or 5 mM and in the presence or absence of 5% glycerol did not stabilize the activity. Although monothioglycerol stabilizes α-KIC oxygenase, it decreases the initial activity, because α-KIC oxygenase is inhibited by assay concentrations of monothioglycerol greater than 0.6%, possibly due to a depletion of O₂ in the assay mixture. This can be prevented by restricting the amount of monothioglycerol introduced into the assay to below 0.2%.

Purification of α-Ketoisocaproate Oxygenase Activity—Table I summarizes a purification of α-KIC oxygenase using conditions which stabilize this enzyme. The final steps (Steps 6–9), but not the initial ones, contained 5% monothioglycerol. Although the recovery for the concentrated DEAE-pool was only 50% for this preparation, recoveries of 70–80% are usually obtained at this stage. As indicated under "Methods," the phenyl-Sepharose and Sephacryl S-200 columns were pre-treated with DEAE-side fractions and 5% monothioglycerol which increased the yields greatly. The overall yield of α-KIC oxygenase in the Sephacryl S-200 pool was 27% with a final specific activity of 104 nmol/min/mg of protein. This fraction is referred to as the purified α-KIC oxygenase herein.

Molecular Weight of Rat Liver α-Ketoisocaproate Oxygenase—SDS-gel electrophoresis of the purified α-KIC oxygenase showed one major protein band with several minor protein bands (Fig. 2A). When electrophoresis was carried out under nondenaturing conditions, α-KIC oxygenase activity migrated with the major protein band (Fig. 2C).

The subunit molecular weight of α-KIC oxygenase determined by SDS-gel electrophoresis was 46,000 (Fig. 2B). Molecular weight of the α-KIC oxygenase was also determined under nondenaturing conditions using Sephacryl S-200 chro-

| Table I |

Summary of purification of α-ketoisocaproate oxygenase from rat liver

| Fraction | Volume (ml) | Total protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min/mg) | % recovery | Fold change |
|----------|-------------|--------------------|--------------------------|--------------------------------|------------|------------|
| 1. 10,000 x g supernatant | 7,200 | 104,200 | 58,600 | 0.55 | (100) | (1.0) |
| 2. 45–75% (NH₄)₂SO₄ fraction | 1,200 | 34,700 | 43,100 | 1.24 | 74 | 2.2 |
| 3. Pre-DEAE-dialysate | 1,400 | 20,900 | 34,800 | 1.67 | 59 | 3.0 |
| 4. DEAE-cellulose pool | 1,770 | 4,000 | 30,500 | 7.92 | 52 | 13.6 |
| 5. Concentrated DEAE-pool | 250 | 4,200 | 29,200 | 6.95 | 36 | 10.7 |
| 45–75% (NH₄)₂SO₄ fraction | 1,770 | 4,000 | 30,500 | 7.62 | 52 | 13.6 |
| 5. Concentrated DEAE-pool | 250 | 4,200 | 29,200 | 6.95 | 36 | 10.7 |

16 ml of the concentrated DEAE-pool was used for the remainder of the purification

| 6. Pre-phenyl-Sepharose | 120 | 1,800 | 12,400 | 6.87 | 45 | 12.3 |
| 7. Phenyl-Sepharose pool | 675 | 513 | 9,790 | 19.1 | 36 | 34.1 |
| 8. Phenyl pool concentrated | 30 | 474 | 10,700 | 22.6 | 38 | 40.3 |
| 9. Sephacryl S-200 pool | 47 | 71 | 7,452 | 104 | 27 | 106 |

α-KIC oxygenase activity was determined in each fraction. The data were plotted and the molecular weight was determined by the method of Andrews (11).

The percentage of the initial activity remaining in the concentrated DEAE-pool was used for the remainder of the purification.

Addition | α-Ketoisocaproate oxygenase activity | % activity remaining |
|----------|-------------------------------------|---------------------|
| None | | |
| 1 mM dithiothreitol | 26.4 | 11.6 | 6 |
| 5 mM dithiothreitol | 25.5 | 9.48 | 6 |
| 1% monothioglycerol | 25.0 | 7.93 | 6 |
| 5% monothioglycerol | 24.3 | 7.13 | 6 |
| 5% glycerol + 1 mM dithiothreitol | 20.9 | 21.1 | 6 |
| 5% glycerol + 5 mM dithiothreitol | 21.7 | 11.6 | 6 |

All aliquots were adjusted to a final volume of 0.17 ml. α-KIC oxygenase activity was measured as described under "Methods" immediately (Initial activity) and after 6 or 8 days of storage at 4°C (Final activity). Results are means of two replicate assays. Each assay contained 29 μg of protein.

Stability of α-ketoisocaproate oxygenase in the presence of monothioglycerol, dithiothreitol, or glycerol

| Addition | α-Ketoisocaproate oxygenase activity | Initial | Final |
|----------|-------------------------------------|--------|-------|
| None | | |
| 1 mM dithiothreitol | 26.4 | 11.6 | 6 |
| 5 mM dithiothreitol | 25.5 | 9.48 | 6 |
| 1% monothioglycerol | 25.0 | 7.93 | 6 |
| 5% monothioglycerol | 24.3 | 7.13 | 6 |
| 5% glycerol + 1 mM dithiothreitol | 20.9 | 21.1 | 6 |
| 5% glycerol + 5 mM dithiothreitol | 21.7 | 11.6 | 6 |

The subunit molecular weight of α-KIC oxygenase determined by SDS-gel electrophoresis was 46,000 (Fig. 2B). Molecular weight of the α-KIC oxygenase was also determined under nondenaturing conditions using Sephacryl S-200 chro-

Rat Liver α-Ketoisocaproate Oxygenase

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Rat Liver α-Ketoisocaproate Oxygenase

A

18.4

ABCD

OXWSE

B

lo′

012

0′4 016

0:8

10

RM

FIG. 2. Polyacrylamide gel electrophoresis of the α-ketoisocaproate oxygenase using denaturing or nondenaturing conditions. A, results of SDS-polyacrylamide gel electrophoresis of 66 μg (A), 24 μg (B), or 12 μg (C) of the purified α-KIC oxygenase (Sephacryl S-200 pool, Table I). Molecular weight standards in column D and their abbreviations are: PHOS, glycogen phosphorylase a (94,000); BSA, bovine serum albumin (66,000); OVA, ovalbumin (45,000); TRYP, trypsinogen (24,000); LACT, β-lactoglobulin (18,400); and LYS, lysozyme (14,300). B, relative mobilities (Rm) of these standards are plotted against the log of their molecular weights. C, results of native gel electrophoresis of the α-KIC oxygenase. A 20-μl (36 μg of protein) aliquot of the purified α-KIC oxygenase (Sephacryl S-200 pool, Table I) was applied to two separate gels. Protein (upper figure) and α-KIC oxygenase activity (lower figure) were monitored as described under "Methods." LDH, lactate dehydrogenase.

matography, according to the method of Andrews (11). Mr = 51,000 was obtained by this method (Fig. 3).

Storage and Stability of the Purified α-Ketoisocaproate Oxygenase—Purified α-KIC oxygenase was quite stable at 4 °C in the presence of 5% monothioglycerol. Only 15% of the activity was lost over 3 weeks, but by 70 days all of the activity was lost. This may be due to the gradual autooxidation of the monothioglycerol which stabilizes the enzyme.

At −80 °C, the oxidase is stable for at least 70 days. Routinely, small aliquots of the purified α-KIC oxygenase were stored separately at −80 °C and thawed prior to use.

Metal Requirement of the α-Ketoisocaproate Oxygenase—Since Fe2+ is required by the oxygenase, the effects of several metals were tested (Table III). In the presence of ascorbate and dithiothreitol, ferrous iron (FeSO4 and FeCl2) and ferric iron (FeCl3) enhanced the α-KIC oxygenase activity. In the absence of added iron, but with added ascorbate and dithiothreitol the activity was 25% of that in the presence of iron. When ascorbate, dithiothreitol, and iron were omitted, the activity was negligible. o-Phenanthroline, an iron chelator, abolishes most of the α-KIC oxygenase activity even in the presence of ascorbate and dithiothreitol. A large nonenzymatic decarboxylation of α-KIC was obtained in the presence of 1 mM o-phenanthroline. This high rate of nonenzymatic decarboxylation occurred only when both o-phenanthroline and ascorbate were included in the assay mixture.

At 1.0 mM, CoCl2, CaCl2, MgCl2, MnCl2, ZnCl2, CdCl2, and NiCl2 did not affect the activity. HgCl2 and cuprous or cupric copper were inhibitory.

The ability of ferric iron (Fe3+) to replace ferrous iron (Fe2+) in the activation of the α-KIC oxygenase could be due to reduction of Fe3+ to Fe2+ by ascorbate. To test this possibility, the effect of ascorbate and dithiothreitol on α-KIC oxygenase activity in the presence of FeCl2, FeCl3, or no added iron was determined (Fig. 4). In the presence of ascorbate and dithiothreitol, FeCl2 and FeCl3 gave similar results, but in the absence of added iron the activity was reduced 72%. Omission of either ascorbate or dithiothreitol had little effect on the activity in the presence of FeCl2 or FeCl3. However, when both ascorbate and dithiothreitol were omitted, FeCl3, but not

Fig. 3. Molecular weight of the α-ketoisocaproate oxygenase determined by Sephacryl S-200 chromatography. The molecular weight of the purified α-KIC oxygenase (Sephacryl S-200 pool, Table I) was determined by Sephacryl S-200 chromatography as described under "Methods." Molecular weight standards are: BSA, bovine serum albumin (66,000); OVA, ovalbumin (45,000); and Cyt c, cytochrome c (12,400).
**Table III**

**Rat Liver α-Ketoisocaproate Oxygenase**

α-Ketoisocaproate oxygenase was assayed as described under "Methods" except that FeSO₄ was replaced by the addition shown. All assays contained 1 mM dithiothreitol (DTT) and 0.5 mM ascorbate except where indicated. Each assay contained 10 µl (12 µg of protein) of the Sephacryl S-200 pool. Values are the means of two replicate assays. Blank activity was measured in duplicate assays in which the Sephacryl S-200 pool was replaced by 10 µl of 20 mM Tris-HCl, pH 7.8, 1% isopropanol, 0.1 M NaCl, 5% monothioglycerol.

| Addition (or deletion) | α-KIC oxygenase activity | Blank | % of control activity |
|------------------------|--------------------------|-------|-----------------------|
| None                   | 23.5                     | 1.7   | 25                    |
| None (−ascorbate, −DTT)| 1.3                      | 2.2   | 1                     |
| 1 mM FeCl₂             | 92.7                     | 0.6   | 106                   |
| 1 mM FeCl₃             | 99.3                     | 0.6   | 100                   |
| 1 mM CuCl₂             | 19.8                     | 1.6   | 21                    |
| 1 mM CaCl₂             | 15.1                     | 0.8   | 15                    |
| 1 mM MgCl₂             | 24.8                     | 1.5   | 27                    |
| 1 mM CuCl              | 9.4                      | 1.5   | 10                    |
| 1 mM CuSO₄             | 9.7                      | 1.5   | 10                    |
| 1 mM ZnCl₂             | 25.1                     | 1.7   | 27                    |
| 1 mM NiCl₂             | 26.2                     | 1.1   | 28                    |

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**Fig. 4.** Effect of dithiothreitol and ascorbate on activation of the α-Ketoisocaproate oxygenase by FeCl₃ or FeCl₄. The oxygenase activity was measured as described under "Methods" except that FeSO₄ was replaced by either FeCl₃ (1 mM) or FeCl₄ (1 mM). Ascorbic acid (0.5 mM) and dithiothreitol (DTT, 1 mM) were only added where indicated. Each assay contained 25 µl (20 µg of protein) of the purified α-KIC oxygenase. Monothioglycerol was not present in the assays. Values are mean of duplicate assays.

FeCl₃, stimulated the oxygenase. These results suggest that the reduced form of iron, Fe²⁺, is utilized by the enzyme. In the presence of ascorbate or dithiothreitol, Fe³⁺ may be reduced to Fe²⁺.

Previous studies (3) indicated that the α-KIC oxygenase required a sulfhydryl reducing agent for maximum activity; yet only a slight effect of dithiothreitol is apparent in Fig. 4. The samples used for these experiments were isolated in 5% monothioglycerol (Sephacryl S-200 pool, Table I). The monothioglycerol was rapidly removed by passage over a Bio-Gel P-6 column and the enzyme was stored at −80 °C (P-6 pool, see "Methods"). Sulfhydryl groups of the enzyme may remain reduced during this treatment, thus eliminating the requirement for a sulfhydryl reducing agent during the assay.

FeSO₄ gave optimal activation of the α-KIC oxygenase between 1.0 and 5.0 mM. Concentrations above 5 mM became progressively inhibitory. The effect of ascorbate on α-KIC oxygenase activity was tested at suboptimal (0.05 mM) and optimal (2 mM) concentrations of FeSO₄, data not shown. In the presence of 0.05 mM FeSO₄, 1.0 mM ascorbate increased α-KIC oxygenase activity about 1.6-fold, but in the presence of 2 mM FeSO₄, ascorbate had very little effect on the activity. The stimulatory effect of ascorbate, therefore, is probably due to its capacity to keep iron in the reduced, ferrous state.

**Optimal Assay Conditions for the α-Ketoisocaproate Oxygenase**—With purified α-KIC oxygenase, the activity was linear with time for 60 min and with protein concentration (up to 6 µg) (data not shown). The pH optimum of the α-KIC oxygenase was determined using several different buffers (Fig. 5) at constant ionic strength. The pH optimum in a Tris-maleate buffer was 6.0. The activity was much lower when 4-morpholineethanesulfonic acid, 4-morpholinepropanesulfonic acid, or Tris buffers were used. Above pH 7.0 the assay mixtures turned a reddish-brown color, presumably due to oxidation of FeSO₄.

The variability of α-KIC oxygenase activity in different buffers was not an ionic strength effect since increasing the concentration of NaCl from 0.05 to 0.4 M had little effect. In contrast, changing the concentration of maleate from 50 mM to 0.1 mM increased the activity 1.5-fold (Fig. 6). Optimal activity was obtained at 0.1–0.2 M maleate. Higher concentrations were inhibitory.

Maleate may activate the α-KIC oxygenase by forming a chelate of Fe²⁺ which is favorable for the catalytic reaction. EDTA (1.0–5.0 mM) and ADP (1.0–10.0 mM), other iron chelators, were tested in the presence of 50 mM 4-morpholineethanesulfonic acid, a buffer which has very little tendency to bind metal ions (13). These compounds caused considerable inactivation of α-KIC oxygenase activity (data not shown). Therefore, the α-KIC oxygenase either prefers the Fe-maleate complex or maleate activates this enzyme by some other mechanism.

**Fig. 5.** pH Optimum. α-KIC oxygenase activity was assayed as described under "Methods" except that the following buffers were used: 0.1 M Tris, 0.1 M maleic acid (●), 0.2 M 4-morpholineethanesulfonic acid (○); MES) 0.2 M 4-morpholinepropanesulfonic acid (MOPS) (△); or 0.2 M Tris (▲). The pH of the buffers was adjusted with NaOH or HCl. The final ionic strength of all buffers was adjusted to 0.47 with NaCl. Each assay contained 10 µl (12 µg of protein) of the Sephacryl S-200 pool (Table I). Values are mean of duplicate assays.
**Substrate Specificity of the α-Ketoisocaproate Oxygenase**

Crude preparations of rat liver cytosol (3) oxidatively decarboxylate both α-KIC and α-keto-γ-methylbutyrate. To determine whether both α-keto acids are decarboxylated by the same enzyme, decarboxylation of α-[1-14C]KIC and α-[1-14C]keto-γ-methylbutyrate was monitored in the various purification fractions (Table IV). The ratio of decarboxylation of α-KIC to that of α-keto-γ-methylbutyrate was approximately 1.0 and did not vary significantly throughout the purification, indicating that one enzyme catalyzes the decarboxylation of both substrates.

The apparent $K_m$ values of the α-KIC oxygenase for α-KIC and α-keto-γ-methylbutyrate were determined using optimized assay conditions (Fig. 7). The apparent $K_m$ for α-KIC was 0.32 ± 0.02 mM. The apparent $K_m$ for α-keto-γ-methylbutyrate was 1.90 ± 0.12 mM. In contrast, the $V_{max}$ for α-keto-γ-methylbutyrate is higher than the $V_{max}$ with α-KIC as the substrate (247 ± 6 versus 130 ± 3 nmol/min/mg of protein).

**Product Identification**—Previous studies (3) indicated that β-hydroxyisovaleryl-CoA is the major product of the reaction. However, when α-[4,5,13H]KIC was incubated with a partially purified preparation of oxygenase several radioactive compounds were detected. Thus, the purified α-KIC oxygenase was incubated with α-[U-14C]KIC and the products were separated as previously described (3). Two peaks containing radioactivity were obtained by Dowex-1-chloride chromatography (solid line, Fig. 8). The major peak (Peak I) was identified as β-hydroxyisovaleryl-CoA by gas chromatography as previously described (3). Peak III, present in small amounts, migrates similarly to isovaleryl acid. Isovaleric acid, however, is not a free intermediate of this enzymatic reaction (3). In the absence of enzyme (dotted line, Fig. 8) all of the radioactivity remained in the α-KIC peak.

**DISCUSSION**

Although our enzyme preparation contained small amounts of about 5 proteins, the major protein band migrated with the α-KIC oxygenase activity during native polyacrylamide gel electrophoresis. This protein oxidatively decarboxylated and hydroxylated α-KIC to form β-hydroxyisovaleryl-CoA. Previous studies demonstrated a requirement for molecular oxygen (3). Determination of molecular weight using denaturing and non-
denaturing conditions indicated that the enzyme is a monomer with $M_r \approx 50,000$.

The activation of the $\alpha$-KIC oxygenase by ferrous iron ($\text{Fe}^{2+}$), ascorbate (NADH or NADPH), and a sulfhydryl compound is typical of many non-heme iron-requiring oxygenases (14). Although other metals did not substitute for $\text{Fe}^{2+}$, ferric iron ($\text{Fe}^{3+}$) was as effective as $\text{Fe}^{2+}$ in the presence of either ascorbate or diithiothreitol. This activation was due to the reduction of $\text{Fe}^{3+}$ to $\text{Fe}^{2+}$ by the reducing agents. The stimulatory effect of ascorbate on the $\alpha$-KIC oxygenase appears to be due to reduction of iron to the ferrous state ($\text{Fe}^{2+}$) since ascorbate had no effect in the presence of high concentrations of $\text{Fe}^{3+}$.

Previously we demonstrated that the $\alpha$-KIC oxygenase requires a sulfhydryl compound, such as diithiothreitol or CoASH (3). The data in Table II show that monothioglycerol, at a concentration of 0.6 M (5%), stabilizes the purified enzyme when stored at 4 °C. The stabilization of the $\alpha$-KIC oxygenase by monothioglycerol may not be entirely due to its ability to reduce enzyme sulfhydryl groups since 0.12 M monothioglycerol should be an adequate sulfhydryl reducing agent, yet it does not prevent $\alpha$-KIC oxygenase inactivation. The high concentration of monothioglycerol (0.6 M) may protect the enzyme from inactivation by oxygen. Many dioxygenases are inactivated by oxygen (14, 15).

Optimal activity of the purified $\alpha$-KIC oxygenase occurs at pH 5.0. The low pH optimum for our assay conditions may be artifactual since $\text{Fe}^{3+}$ is rapidly oxidized at higher pH to ferric hydroxide. With reactions carried out at pH 7.0 or greater, the assay mixtures developed a reddish-brown color which was and ADP did not replace maleate and actually inhibited the hydroxide. With reactions carried out at pH 7.0 or greater, the concentration from 50 to 2 M gave almost a 2-fold increase in the $\alpha$-KIC oxygenase activity which apparently was not an ionic strength effect since varying the concentrations of NaCl had no effect on the $\alpha$-KIC oxygenase activity (5). It is possible that maleate forms a complex with $\text{Fe}^{3+}$ favorable for catalysis. Other metal chelators such as EDTA and ADP did not replace maleate and actually inhibited the $\alpha$-KIC oxygenase.

The cytosolic $\alpha$-KIC oxygenase also uses $\alpha$-keto-$\gamma$-methiolbutyrate (the $\alpha$-ketanalog of methionine) as a substrate. The affinity of the enzyme for $\alpha$-keto-$\gamma$-methiolbutyrate (apparent $K_m = 1.9 \text{ mm}$) is less than that for $\alpha$-KIC (apparent $K_m = 0.3 \text{ mm}$), but the $V_{max}$ is greater. The product formed from $\alpha$-keto-$\gamma$-methiolbutyrate has not yet been identified, but it migrates identically to $\beta$-hydroxyisovalerate using Dowex-1-chloride chromatography.2 If the reaction is similar to the $\alpha$-KIC oxygenase reaction, the expected product would be $3$-hydroxy-3-methylthiopropionic acid. The product of the $\alpha$-KIC oxygenase reaction, the expected product would be $3$-hydroxyisovaleryl-CoA. This is consistent with the results presented here and indicates the activity could be of importance when methionine or $\alpha$-keto-$\gamma$-methiolbutyrate levels are elevated, such as in hypermethioninemia (45). The mitochondrial $\alpha$-keto-$\gamma$-methiolbutyrate decarboxylase has a $K_m$ of 0.1–0.6 mm for this substrate (44), but the activity apparently is due to the branched-chain $\alpha$-keto acid dehydrogenase (46). The utilization of $\alpha$-keto-$\gamma$-methiolbutyrate by the same cytosolic and mitochondrial enzymes that metabolize $\alpha$-KIC is interesting, especially since rat liver also contains an enzyme that transaminates both leucine and methionine to form these $\alpha$-keto acids (47).

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