Absence of 5-Hydroxy-4-Ketohexanoate and the α-Ketoglutarate Dehydrogenase Complex in Mutants of Saccharomyces oviformis Incapable of Growing on Ethanol

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The roles of the enzyme which forms 5-hydroxy-4-ketohexanoate (HKH) and of related enzymes in the metabolism of ethanol were studied in Saccharomyces oviformis WH92 and its mutants, which grew poorly or not at all on ethanol. The strains, which did not grow on ethanol, did not form HKH from α-ketoglutarate and acetaldehyde enzymatically and were also devoid of the α-ketoglutarate dehydrogenase complex. Acetaldehyde inhibited the activity of α-ketoglutarate dehydrogenase. These mutants did not grow on acetate since they had no acetyl-CoA synthetase activity. The relationship of the formation of HKH with the metabolism of ethanol is discussed.

In our previous papers (4–6), we demonstrated that certain strains of yeast grow on ethanol and produce 5-hydroxy-4-ketohexanoate (HKH). Moreover, a cell-free extract of Hansenula miso IFO 0146 was found to form HKH enzymatically from α-ketoglutarate and acetaldehyde (7).

This paper deals with the relationship of the enzyme which forms HKH and with the enzymes related to ethanol metabolism in Saccharomyces oviformis.

MATERIALS AND METHODS

Growth of yeast. S. oviformis WH92, which utilizes ethanol as the sole carbon source, was used as the parent strain. Mutants which grew poorly or not at all on ethanol and those which grew on ethanol but not on acetic acid were also selected from mutants of S. oviformis WH92 isolated by Y. Oshima.

Organisms were grown in medium containing (per liter of water) peptone (Difco), 5 g; yeast extract, 5 g; KH₂PO₄ and MgSO₄·7H₂O, 1 g; and various compounds as carbon sources, 10 g. Volatile compounds were filtered through a Seitz filter and then added to the autoclaved solution. There was no detectable growth unless peptone or yeast extract was supplied. Cultures were grown in 5 ml of medium in 25-ml conical flasks which were shaken reciprocally at 120 strokes per min at an amplitude of 7 cm at 30°C for 48 hr. Optical density was measured in a Klett-Summerson colorimeter (filter no. 66).

Preparation of cell-free extracts. Cells were grown in a medium containing glucose as the sole source of carbon for 48 hr at 30°C, harvested by centrifugation at 5,000 x g for 15 min at 5°C, and washed twice with 0.01 M potassium phosphate buffer, pH 7.5. Washed cells were disrupted in a Braun cell homogenizer (model MSK) as follows: 10 g of washed cells was suspended in 10 ml of cold 0.1 M potassium phosphate buffer, pH 7.5. The cooled suspension was put into a cold 75-ml flask containing 50 g of cold beads. The flask was closed with a ground-glass stopper held by a rubber strap, and the flask was inserted into the inner chamber of the homogenizer. The protective cover was closed, and the chamber and flask were cooled for a few seconds with liquid CO₂ and then agitated. The flow of liquid CO₂ to cool the mixture was continued during homogenization at 4,000 cycles per min for 1 min. After centrifugation at 1,000 x g for 10 min, the extract was centrifuged at 28,000 x g for 30 min at 5°C, and the supernatant was used as the cell-free extract. Its protein concentration was about 25 mg per ml as estimated by the Lowry method (13).

Enzyme assays: α-Ketoglutarate dehydrogenase activity. α-Ketoglutarate dehydrogenase (EC 1.2.4.2) activity was assayed by a modification of the method described by Schwartz et al. (17), based on the following reaction:

\[
\text{HOOC-CH}_2\text{CH}_2\text{COOH} + 2\text{Fe(CN)}_4^{3-} + \text{H}_2\text{O} \xrightarrow{\text{TTP}} \text{HOOC-CH}_2\text{CH}_2\text{COOH} + \text{CO}_2 + 2\text{Fe(CN)}_4^{4-} + 2\text{H}^+
\]

The routine assay mixture contained 100 mM potassium phosphate buffer (pH 7.5), 40 mM sodium
α-ketoglutarate, 20 mM MgCl₂, 0.2 mM thiamine pyrophosphate (TPP), 1.88 mM potassium ferricyanide, and enzyme solution (1 mg of protein) in a total volume of 3 ml. Reduction of ferricyanide was monitored at 420 nm with a Hitachi recording spectrophotometer, model 124, at 25 C. Enzyme activity is expressed as micromoles of ferricyanide reduced per minute.

α-Ketoglutarate decarboxylase activity. α-Ketoglutarate decarboxylase (EC 4.1.1.1) activity was assayed as follows. A mixture of 80 mM potassium phosphate buffer (pH 7.5), 40 mM sodium α-ketoglutarate, 90 mM acetaldehyde, 20 mM MgCl₂, and 0.2 mM TPP was placed in the main chamber of a Warburg vessel with enzyme solution in the side arm. The total volume was 2 ml. After equilibration at 30 C, the enzyme was added to the main compartment and the rate of carbon dioxide evolution was determined.

HKH-forming and acetoin-forming activities. Unless otherwise mentioned, the standard mixture for assay consisted of enzyme solution (2 mg of protein), 40 mM sodium α-ketoglutarate (for HKH-forming activity) or sodium pyruvate (for acetoin-forming activity), 90 mM acetaldehyde, 20 mM MgCl₂, 0.2 mM TPP, and 100 mM potassium phosphate buffer (pH 7.5) in a total volume of 1.0 ml. The reaction mixture was incubated for 1 hr at 30 C. A linear relationship between the activity and time was obtained with incubation periods of up to 1 hr, as mentioned before (7).

The reaction was stopped by addition of 0.1 ml of 4 N perchloric acid, and the mixture was centrifuged. The acetoin or HKH formed in the reaction mixture was separated by the method of Bloom et al. (2). The supernatant was adjusted to pH 7.0 with 1 N potassium hydroxide and passed through a column (8 by 45 mm) of well-washed Dowex 1-x10, formate form. Then 10 ml of water was passed through the column to wash out all the acetoin. The effluents were combined, and a sample was used for colorimetric determination of acetoin. HKH was eluted quantitatively by passing 9 ml of 1 M formic acid through the column. Sodium hydroxide (1 ml, 4.5 N) was added to the eluate to neutralize the formic acid, and a sample of this solution was used for colorimetric determination of HKH.

The ketol group in HKH or acetoin reacts with creatine and α-naphthol to give a positive Voges-Proskauer reaction, and HKH and acetoin can thus be determined colorimetrically as described previously. Creatine (1 ml of 5% solution) was mixed with 5 ml of test solution containing 1 to 100 μg of HKH or acetoin. α-Naphthol (1 ml of 5% solution) was added, and the color was allowed to develop at room temperature for 90 min with occasional shaking. It was then read against a reagent blank in a Klett-Summerson colorimeter (filter no. 54).

Alcohol dehydrogenase activity. The assay of alcohol dehydrogenase (EC 1.1.1.1) is based on the rate of conversion of ethanol to acetaldehyde. The assay mixture contained (as final concentrations) 100 mM ethanol, 3 mM nicotinamide adenine dinucleotide (NAD), 100 μM tris (hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.5) and enzyme (mg of protein) in a total volume of 1.0 ml. The assay was performed at 37 C for 20 min. The activity was on the linear portion of the curve up to 30 min. The amount of acetaldehyde formed was determined colorimetrically as the 2,4-dinitrophenylhydrazine by a modification of the method of Ariga et al. (1).

Acetyl-CoA synthetase activity. Acetyl-CoA synthetase (EC 6.2.1.1) activity was assayed by a modification of the method of Jones and Lipmann (9). The assay mixture contained (as final concentrations) 81 μM CoA, 10 mM adenosine triphosphate, 20 mM potassium acetate, 100 mM potassium phosphate buffer (pH 7.5), 200 mM hydroxylamine solution, 50 mM potassium fluoride, 10 mM MgCl₂ and 10 mM glutathione and water, if necessary, to give a volume of 1 ml after addition of the enzyme. The tubes were placed in a bath at 37 C for 5 min before the enzyme was added. After addition of the enzyme, the tubes were incubated for 20 min, and 1.5 ml of ferric chloride reagent, containing 10% FeCl₃·6H₂O and 3.3% trichloroacetic acid in 0.66 N HCl, was added. The acetylhydroxamate formed was measured in a Klett-Summerson colorimeter (no. 54 filter) by using a setting of zero for the absorption of a mixture of 1 ml of water and 1.5 ml of ferric chloride reagent. A blank containing no CoA was always included with the tubes containing CoA, and the blank reading was subtracted from the reading obtained when CoA was present.

Chemicals. All chemicals used were of the highest commercial quality available.

RESULTS

Growth of Saccharomyces oviformis WH92 and mutant strains with various carbon sources. The abilities of the parent and mutant strains to grow in media containing various carbon sources were tested (Table 1). None of the strains grew in media containing any member of the tricarboxylic acid cycle, α-ketoglutarate, succinate, malate, or citrate. WH92-5A grew only in media containing ethanol and glucose. WH92-17 and T27-8D did not grow in medium containing acetate but, unlike the parent strain, grew slightly in ethanol. T29-10A grew only in media containing lactate and glucose. T4-4B and T610D grew only in a medium containing glucose. It is possible that members of the tricarboxylic acid cycle could not be used as the sole carbon source by these strains because they did not penetrate into the cells, as mentioned by Folkes (3).

HKH-forming activity and related enzyme activities in parent and mutant strains. The HKH-forming activity and related enzyme activities were measured in the parent and mutant strains described above.

Previous papers showed that HKH was formed by enzymatic condensation of acetaldehyde with α-ketoglutarate. In this reaction, succinic semialdehyde, formed by decarboxylation of α-keto-
glutarate, may react with acetaldehyde to form HKH. $\alpha$-Ketoglutarate decarboxylase activity is known to be associated with $\alpha$-ketoglutarate dehydrogenase (12). Thus, the HKH-forming activity, $\alpha$-ketoglutarate decarboxylase, and $\alpha$-ketoglutarate dehydrogenase were measured in the parent and mutant strains (Table 2). In these experiments, cells grown in glucose medium were used, since glucose is the only carbon source with which all the strains tested could grow. The activities were all high in the parent strain and WH92-5A, which grew well on ethanol. These activities were not detectable in T29-10A, T4-4B, and T6-10D which could not grow on ethanol. Rather low activities were found in WH92-17 and T27-8D which grew slightly on ethanol. These results suggest that the $\alpha$-ketoglutarate dehydrogenase complex is required for aerobic utilization of ethanol but is not essential for utilization of glucose, although it is uncertain if the $\alpha$-ketoglutarate dehydrogenase complex; hence, it is uncertain whether formation of HKH is essential for ethanol utilization. Acetaldehyde, another compound involved in HKH formation, is known to condense with pyruvate to form acetoin in yeast (12). The enzyme which forms acetoin differs from that forming HKH, as shown previously (4). None of the mutant strains tested could grow in acetate medium. Thus, acetyl-CoA synthetase, alcohol dehydrogenase, and the acetoin-forming enzyme were examined in the parent and mutant strains (Table 3). The activities of the acetoin forming enzyme were higher in the mutant strains than in the parent strain, indicating that the enzyme is not related to the utilization of ethanol. The activities of alcohol dehydrogenase, the first enzyme in the pathway of ethanol utilization, varied in different strains. WH92-5A which grew well on ethanol had higher enzyme activities than other mutant strains. None of the mutant strains which did not grow on acetate had any acetyl-CoA synthetase activity. It is possible that these mutants could not utilize acetate because they lacked acetyl-CoA synthetase.

**Effect of acetaldehyde on HKH-formation and related enzyme actions.** Table 4 shows the activi-

### Table 1. Utilization of various carbon sources by *Saccharomyces oviformis* WH92 and its mutants

| Carbon source | WH92 | WH92-5A | WH92-17 | T27-8D | T29-10A | T6-10D |
|---------------|------|---------|---------|--------|---------|--------|
| Ethanol       | 1.80 | 0.20    | 0.24    | 0.16   | 0       | 0      |
| Acetic acid   | 2.00 | 0.00    | 0.00    | 0      | 0       | 0      |
| Pyruvic acid  | 0.74 | 0.62    | 0.60    | 0      | 0       | 0      |
| Lactic acid   | 1.44 | 1.22    | 1.60    | 0.56   | 0       | 0      |
| Glycerol      | 2.88 | 3.04    | 0.72    | 0      | 0       | 0      |
| Glucose       | 3.26 | 2.74    | 2.30    | 0.72   | 0.80    | 1.00   |

* Optical densities of cultures of each strain in basal medium were ca. 0.03. The optical density shown is the optical density with the test carbon source minus the optical density in basal medium.

### Table 2. Activities of 5-hydroxy-4-ketohexanoate formation and of $\alpha$-ketoglutarate dehydrogenase and $\alpha$-ketoglutarate decarboxylase

| Mutants   | HKH-forming activity (HKH formed)* | $\alpha$-Ketoglutarate dehydrogenase activity (tetracyanide reduced)* | $\alpha$-Ketoglutarate decarboxylase activity (CO$_2$ evolved)* |
|-----------|-----------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| WH92      | 193                               | 15                                                           | 3.9                                                           |
| WH92-5A   | 221                               | 17                                                           | 4.1                                                           |
| WH92-17   | 36                                | 1.7                                                          | 0.4                                                           |
| T27-8D    | 41                                | 2.5                                                          | 0.6                                                           |
| T29-10A   | 0                                 | 0                                                            | 0                                                             |
| T4-4B     | 0                                 | 0                                                            | 0                                                             |
| T6-10D    | 0                                 | 0                                                            | 0                                                             |

* Values expressed as micromoles per hour per milligram of protein.

### Table 3. Activities of the acetoin-forming enzyme, alcohol dehydrogenase, and acetyl-CoA synthetase in *Saccharomyces oviformis* and its mutants

| Mutants   | Acetoin-forming activity (acetoin formed)* | Alcohol dehydrogenase activity (acetaldehyde formed)* | Acetyl-CoA synthetase activity (acetyl-CoA formed)* |
|-----------|--------------------------------------------|------------------------------------------------------|----------------------------------------------------|
| WH92      | 48                                         | 216                                                  | 2.52                                               |
| WH92-5A   | 70                                         | 213                                                  | 0                                                  |
| WH92-17   | 170                                        | 99                                                   | 0                                                  |
| T27-8D    | 94                                         | 111                                                  | 0                                                  |
| T29-10A   | 150                                        | 60                                                   | 0                                                  |
| T4-4B     | 175                                        | 63                                                   | 0                                                  |
| T6-10D    | 105                                        | 141                                                  | 0                                                  |

* Values expressed as micromoles per hour per milligram of protein.

### Table 4. Activity of HKH-formation in the presence of acetaldehyde

| Mutants   | HKH-forming activity (HKH formed)* | Acetaldehyde dehydrogenase activity (tetracyanide reduced)* | Acetyl-CoA synthetase activity (acetyl-CoA formed)* |
|-----------|-----------------------------------|---------------------------------------------------------------|----------------------------------------------------|
| WH92      | 193                               | 15                                                           | 3.9                                               |
| WH92-5A   | 221                               | 17                                                           | 4.1                                               |
| WH92-17   | 36                                | 1.7                                                          | 0.4                                               |
| T27-8D    | 41                                | 2.5                                                          | 0.6                                               |
| T29-10A   | 0                                 | 0                                                            | 0                                                 |
| T4-4B     | 0                                 | 0                                                            | 0                                                 |
| T6-10D    | 0                                 | 0                                                            | 0                                                 |

* Values expressed as micromoles per hour per milligram of protein.
ties of α-ketoglutarate decarboxylase and dehydrogenase and the rates of HKH formation in the presence and absence of acetaldehyde. Addition of acetaldehyde greatly increased the activity of α-ketoglutarate decarboxylase and resulted in the formation of HKH, whereas the addition of ferricyanide caused an increase in the activities of α-ketoglutarate dehydrogenase and α-ketoglutarate decarboxylase. Thus, in this organism, α-ketoglutarate decarboxylase operates in the presence of an appropriate amount of acetaldehyde, but α-ketoglutarate dehydrogenase does not. The relationship between the amount of acetaldehyde added and its inhibition of α-ketoglutarate dehydrogenase is shown in Fig. 1. It is probable that succinic semialdehyde TPP which may be formed by decarboxylation of α-ketoglutarate (Fig. 2) is converted to HKH in the presence of an appropriate amount of acetaldehyde.

**DISCUSSION**

The present experiments show that the mutants of *S. oviformis* which could not grow on ethanol had no HKH-forming activity, α-ketoglutarate dehydrogenase, or decarboxylase, but contained the acetoin-forming enzyme and alcohol dehydrogenase. A condensation of α-ketoglutarate and acetaldehyde to form HKH was also found in studies with mammalian tissues (2). α-Ketoglutarate decarboxylase is known to be associated with the α-ketoglutarate dehydrogenase complex (12, 15). The complex catalyzes the following overall reaction:

\[
\text{α-Ketoglutarate} + \text{NAD}^+ + \text{CoA-SH} \rightarrow \\
\text{Succinyl-S-CoA} + \text{NADH} + H^+ 
\]

Thus, it occupies an important place in metabolism and is likely to be under metabolic regulation. This reaction represents the main pathway of α-ketoglutarate oxidation in animal tissues, and it also occurs widely in microorganisms. The enzyme system which catalyzes the above reaction has been isolated as a multi-enzyme complex with a molecular weight of several million from pig heart muscle (8, 15), *Escherichia coli* (12), and bakers’ yeast (14). More recently, Schlossberg et al. (16) demonstrated that the α-ketoglutarate dehydrogenase complex in beef heart is responsible for the condensation reaction between α-ketoglutarate and either acetaldehyde or glyoxylate. We also obtained evidence that the α-ketoglutarate dehydrogenase complex from bakers’ yeast has HKH-forming activity (Hirabayashi and Ohmori, unpublished data).

Thus, α-ketoglutarate decarboxylase, involved in the α-ketoglutarate dehydrogenase complex,

![Inhibition of α-ketoglutarate dehydrogenase activity by acetaldehyde.](http://aem.asm.org/)

**Table 4. Effect of acetaldehyde on the activities of α-ketoglutarate decarboxylase and dehydrogenase and 5-hydroxy-4-ketohexanoate formation in Saccharomyces oviformis WH92**

| Additions          | HKH formation (μmoles per hr per mg of protein) | CO₂ evolved (μliter per 10 min per mg of protein) | Ferricyanide reduction (μmoles per min per mg of protein) |
|-------------------|-----------------------------------------------|--------------------------------------------------|-----------------------------------------------------------|
| None...............| 0                                             | 0                                               | 0                                                         |
| Acetaldehyde......| 187                                           | 3.9                                             | 14                                                        |
| Ferricyanide......| 0                                             | 4.1                                             | 14                                                        |
| Acetaldehyde plus ferricyanide......| 180                                           | 3.9                                             | 0                                                         |

* Reaction mixtures contained: 80 mM potassium-phosphate buffer (pH 7.5), 40 mM sodium α-ketoglutarate, 20 mM MgCl₂, 0.2 mM TPP and enzyme solution; 90 mM acetaldehyde and 1.88 mM potassium ferricyanide were added.

**Fig. 1. Inhibition of α-ketoglutarate dehydrogenase activity by acetaldehyde.**

**Fig. 2. Relationship between 5-hydroxy-4-ketohexanoate formation and the α-ketoglutarate dehydrogenase complex.**
may also cause the condensation of α-ketoglutarate and acetaldehyde in S. oviformis. In this reaction, α-ketoglutarate seems to be converted first to succinic semialdehyde TPP. The complex may be required for growth of the organism on ethanol, since mutants which can not grow on ethanol lack this complex.

No acetyl-CoA synthetase was found in WH92-5A and those mutants which could not utilize acetate. When grown on ethanol, it is probable that the organism converts ethanol to acetyl-CoA via acetaldehyde, as shown in Streptococcus faecalis by Kamihara (11).

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