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Threonine is the best substrate for D-lactate formation in octopus tentacle

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Summary. Carbon sources for D-lactate and enzyme activities related to D-lactate formation were investigated using cell-free homogenates of *Octopus vulgaris* tentacle tissue. The results are as follows: a) The best precursor for D-lactate formation was threonine and second best precursors were glycine and fructose-1,6-bisphosphate. Threonine and glycine served as precursors only in presence of glutathione. b) Both amino acids were precursors for methylglyoxal from which D-lactate was synthesized. Alanine, cysteine and serine were not precursors. We present a metabolic map for D-lactate formation in octopus in order to explain these experimental results.

Keywords: Threonine – Glycine – D-Lactate – Methylglyoxal – Octopus

Introduction

The biochemistry of D-lactate has not been thoroughly investigated. The results that we have obtained about its physiological meaning were shortly reviewed in a previous paper (Ohmori et al., 1997). Thus far we have established the following: a) Lactic acid in *Octopus vulgaris* is almost always in the D-form. b) D-Lactate concentration in the octopus is 380 times higher than that in rat skeletal muscle and also 6.4 times higher than the concentration of L-lactate in rat muscle. c) D-Lactate is effectively biosynthesized from pyruvate, methylglyoxal and S-lactoylglutathione in the homogenate of the octopus tentacle muscle. d) Enzyme activity of L-lactate dehydrogenase (L-LDH), D-lactate dehydrogenase (D-LDH), glyoxalase I and II, aldolase, pyruvate kinase, phosphofructokinase and glycerol kinase were assayed in octopus and rat tissues. These enzyme activities in octopus were similar to those in rat skeletal muscle except for the D-LDH and L-LDH activity which was scarcely detected in the octopus. e) D-Lactate was formed from both the methylglyoxal bypass and the Embden Meyerhof pathway in octopus. As the next problem, we wanted to know what are carbon sources for D-lactate other than pyruvate and methylglyoxal as direct precursors in octopus.

Materials and methods

Chemicals

Lithium D-lactate, lithium L-lactate, fructose-1,6-bisphosphate, EDTA-2Na, TRIS, acetyl-CoA, *o*-dianisidine were purchased from Sigma Chemical Co. (St. Louis, MO). D-Lactate dehydrogenase (D-LDH) from *Staphylococcus sp*. and diaphorase from *Clostridium kluiveri* were kindly supplied by Amano Pharmaceutical (Nagoya, Japan). ATP, β-NADH, β-NAD^+, β-NADPH were obtained from Oriental Yeast (Tokyo, Japan). DL-6,8-Thioctamide, *o*-phenylenediamine, and ethyl acetoacetate were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). To prepare acetoacetate solution, 1.3 ml ethyl acetoacetate was hydrolyzed at 40°C for 1 h in 1.5 ml 7 M sodium hydroxide and the hydrolysate was brought to pH 7 with 1 M HCl. Alanine, cysteine, serine and magnesium sulfate were from Katayama Chemical (Osaka, Japan). F-kits for determination of L-lactic acid and glycerol were purchased from Boehringer Mannheim Co., Ltd. (Mannheim, Germany). Methylglyoxal was prepared just before use by hydrolysis of the dimethylacetal (Aldrich, Milwaukee, Inc., USA) (Kellum et al., 1978). 2-Hyroxy-3-methylquinoxaline was prepared from 440 mg sodium pyruvate and 900 mg o-phenylene-diamine 2HCl in 40 ml 2 M HCl at room temperature overnight. The reaction mixture was extracted with three 30 ml portions chloroform. Crystals were twice recrystallized from ethanol. The pale yellow needles (220 mg) had a Rf value of 0.85 on silica gel in n-butanol, acetic acid, water (4:1:1, v/v/v). A kit, Glucose-CII-Test-Wako and other reagents were products of Wako Pure Chemicals (Osaka, Japan).

Animals

Octopuses (*Octopus vulgaris*) with body weight from 1 to 1.5 kg were purchased from a market. They were captured in the Inland Sea in the early morning several hours before the experiment and brought to the laboratory in an ice box. Male, 5-week-old Wistar strain albino rats were obtained from Shimizu Experimental Animal (Kyoto).

Instruments

D-Lactate and methylglyoxal were determined by partially modified methods of Ohmori and Iwamoto (1988) and Ohmori et al. (1987), respectively.

For determination of D-lactate and methylglyoxal, a Gaschro-Kogyo model 576-1A liquid chromatograph (Osaka, Japan) equipped with a Gaschro-kogyo model 502T detector was used. The column was a 150 × 4.6 mm I.D. Cosmosil 5C-18 AR-II (Nacalai Tesque, Kyoto, Japan). The column was eluted with 10 mM potassium phosphate (pH 2.1) containing 15% acetonitrile for D-lactate or containing 20% acetonitrile for methylglyoxal at a flow-rate of 1.0 ml/min. The quinoxaline derivatives were monitored at 334 nm and column temperature was 45°C. Spectrometry was performed with a Shimazu double-beam UV spectrometer (Kyoto, Japan).

Homogenate preparation for formation of D-lactate and methylglyoxal

The tentacles from live octopuses were amputated and cut into small pieces with a scissors. The foot muscle slices were homogenized at 0°C and 10,000 rpm for 5 min with a Waring blender in 5 volumes of 50 mM potassium phosphate (pH 7.0). The homogenates were centrifuged at 4°C and 6,000 × *g* for 15 min. The supernatant was dialyzed at 4°C for 2 h against 5 l of 50 mM potassium phosphate (pH 7.0). The reaction mixture consisted of 50 mM potassium phosphate (pH 7.0), 0.5 mM substrate, 0.2 mM of cofactor and 0.4 ml of the dialyzed supernatant in a final volume of 2.0 ml (further details of these reaction mixtures are shown in Table 1). The mixture was incubated at 25°C for 30 min. Control samples consisted of the dialyzed supernatant without substrate and cofactor and were incubated at 0°C. After incubation, 3 ml methanol was added to the reaction mixture, which was then centrifuged at 3,000 × *g* for 15 min. The supernatant was used for further analysis.

Determination of metabolites

Methylglyoxal was determined according to the method of Ohmori et al. (1987). Briefly, 1 ml of the reaction mixture, 1 ml 1 M perchloric acid and 0.1 ml 1% *o*-phenylenediamine were reacted at room temperature for 1 h. Then 0.2 ml 5 M NaOH was added, and the reaction mixture was extracted with 3 ml dichloromethane. The organic phase (1 ml) was mixed with 0.05 ml 6 M HCl and evaporated using a Savant Speed Vac concentrator (Model SVC-100H, New York, NY, USA) at room temperature. The residue was dissolved in 0.2 ml the column eluant of which 10 μl was subjected to HPLC. D-Lactate was converted by D-LDH into pyruvate, which was further converted into 2-methylquinoxalinol by *o*-phenylenediamine in a one-vial reaction (Ohmori and Iwamoto, 1988). The quinoxalinol was extracted with two portions of 2 ml ethyl acetate. The ethyl acetate extract was evaporated to dryness. The residue was dissolved into 0.2 ml the column equilibration solution. An aliquot (10 μl) was analysed by HPLC. L-Lactate and glycerol were determined with UV methods using the Boehringer F-kit. Glucose was determined by a Glucose-CII-Test-Wako. Glycine was colorimetrically measured by the method of Ohmori (Ohmori et al., 1978). L-Threonine was oxidatively degraded by periodic acid to acetaldehyde which was converted into 2,4-dinitrophenylhydrazone. The hydrazone was determined by gas chromatography. Details of this method will be reported in the near future. Glycine was spectrometrically determined by our method (Ohmori et al., 1978).

Enzyme assays

For the assay for phosphofructokinase, pyruvate kinase, aldolase and glycerol kinase, tissues were treated as follows: the octopus tentacles and rat skeletal muscles were cut into small pieces with a scissors and homogenized at 4°C and 10,000 rpm for 5 min with a Waring blender in 4 vol. of ice-cold 50 mM TRIS-HCl (pH 7.4) containing 0.1 mM KCl, 5 mM magnesium sulfate and 1 mM EDTA. The rat livers were homogenized at 4°C for 1,000 × *g* for 30 sec with a Teflon homogenizer. The homogenates were centrifuged at 4°C and 8,000 × *g* for 10 min and the supernatants were used as enzyme sources. For the assay for glyoxalases I and II, the organs were homogenized in 5 vol. 10 mM potassium phosphate (pH 7.0) and centrifuged as described above. For the activity tests of threonine dehydrogenase (threonine-3-dehydrogenase, TDH) and amine oxidase the tissues were homogenized in 5 vol. 50 mM of potassium phosphate (pH 7.0). The activities of phosphofructokinase, pyruvate kinase, aldolase, glyoxalase I, II, glycerol kinase, TDH and amine oxidase were assayed by the methods of Kemp (1975), Valentine and Tanaka (1966), Gracy et al. (1970), Racker (1955), Bergmeyer et al. (1983), and Ray and Ray (1985, 1987), respectively.

Results and discussion

D-Lactate formation from several simple substances

Glucose, fructose-1,6-bisphosphate, glycerol, acetone, acetoacetate, L-threonine, glycine, alanine, cysteine and serine were chosen as potential precursors based upon known metabolic path and interactions as discussed in previous reports (Kondoh et al., 1994 and Ohmori et al., 1997). They were added to the dialyzed octopus tentacle homogenate and incubated at 25°C for 30 min. The composition of the reaction mixtures is shown in Table 1. The amount of D-lactate formed in the reaction mixtures was determined. As shown in Fig. 1, the striking aspect of the results is that threonine was the best precursor for D-lactate. However, threonine was a poor precursor in rat liver homogenate and perfused rat liver (Kondoh et al., 1994). The second best precursors were fructose-1,6-bisphosphate and glycine. It was also surprising that glycine should be a good precursor. Although glucose is far removed from D-lactate in metabolic map, it still served as a precursor. Previously, we reported that glycerol was the best substrate for D-lactate formation in rat liver (Kondoh et al., 1994), but in the octopus homogenate, it was only a moderately good substrate (Fig. 1). In the mammalian liver

Table 1. Substrates and cofactors added to the reaction mixtures to form D-lactate

Substrate	Cofactor
Glucose	ATP, Mg^{++}
Glycerol	ATP, Mg^{++}, NAD^+, Nicotinamide
Fructose-1,6-diphosphate	ATP, Mg^{++}
Acetone	NADPH, NAD^+, Nicotinamide
Acetoacetate	NADPH, NAD^+, Nicotinamide
Glycine	Acetyl-CoA
L-Threonine	NAD^+, Nicotinamide
methylglyoxal	
S-lactoylglutathione	

Substrates and cofactors were added to the 6,000 *g* supernatant of the octopus tentacle homogenate. Glutathione was added to the reaction mixtures except when S-lactoylglutathione was used as substrate. The final concentrations of cofactors were 0.2 mM, except that of nicotinamide (an inhibitor of NAD(P) nucleosidase) which was 1.0 mM. The final concentrations of methylglyoxal and S-lactoylglutathine were 0.15 mM and those of other substrates and glutathione were 0.5 mM

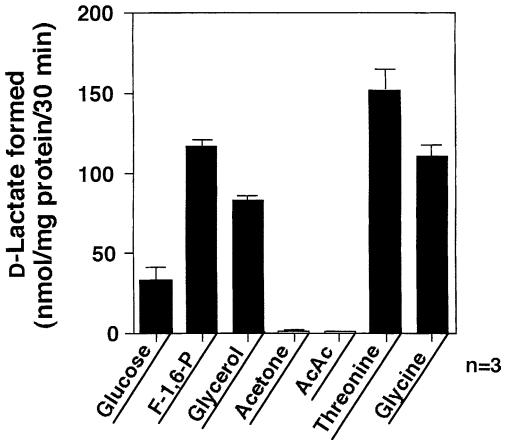

Fig. 1. D-Lactate formation from several precursors in 6,000 × *g* supernatant of octopus tentacle muscle homogenate. The incubation mixtures are shown in Table 1. D-Lactate was measured as described in "Materials and methods". means ± SE, n = 3

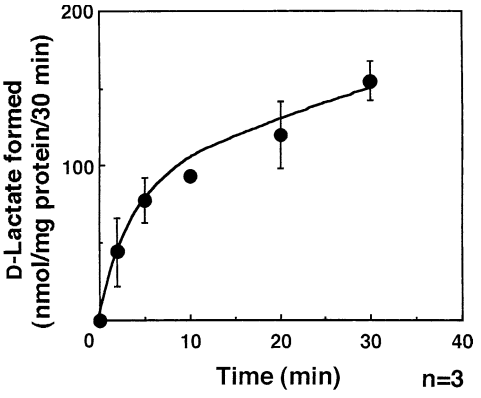

Fig. 2b. Time course of D-lactate formation from 0.5 mM threonine at 25°C in 6,000 × *g* supernatant of homogenate of octopus tentacle muscle. The cofactors are written in Table 1. D-Lactate was measured as 2-methylquinoxalinol by HPLC. means ± SE (n = 3)

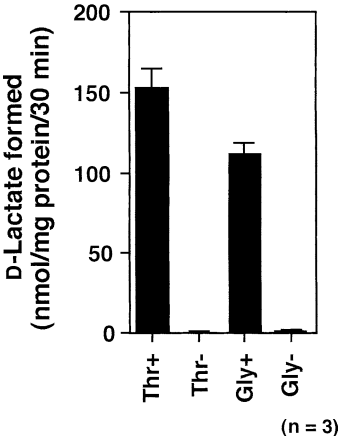

Fig. 3. D-Lactate formation from threonine and glycine with (+) and without (−) glutathione for 30 min at 25°C in 6,000 × *g* supernatant of homogenate of octopus tentacle muscle. The reaction condition and determination method are described in Fig. 2b. Results are means ± SE (n = 3)

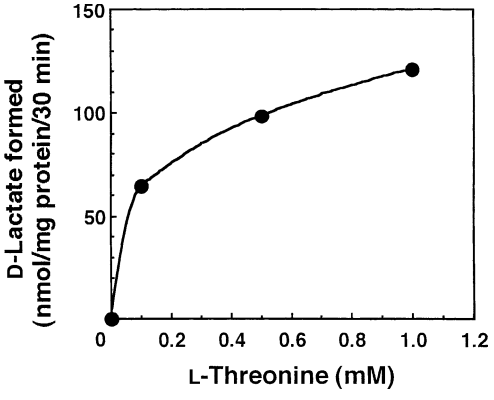

Fig. 2a. The amount of D-lactate formed from threonine after 30 min at 25°C in 6,000 × *g* supernatant of homogenate of octopus tentacle muscle in relation to precursor concentration. The reaction mixture is shown in Table 1. D-Lactate was detected as described in "Materials and methods"

acetoacetate is decarboxylated to acetone and then oxidized to acetol and finally oxidized to methylglyoxal (Milligan and Baldwin, 1967). Acetoacetate and acetone were found to be poor precursors for D-lactate in the rat liver homogenate and perfused rat liver (Kondoh et al., 1994). As shown in Fig. 1, they were also not precursors for D-lactate in the octopus tentacle homogenate. Since L-threonine was utilized most effectively for the D-lactate formation, the concentration dependence of L-threonine as substrate and time dependence on the formation were examined using the tentacle homogenate of octopus. The results are shown in Figs. 2a and b. The D-lactate formation was dependent on the threonine concentration and the reaction time. Figure 3 shows that threonine and glycine could serve as precursors of D-lactate only when they are incubated in the presence of glutathione. Generally, threonine is metabolized through three routes by three enzymes; threonine dehydrogenase (threonine-3-dehydrogenase, TDH), threonine dehydratase (TH) and threonine aldolase (TA). Bird and Nunn (1983) reported that 87% of threonine was degraded by TDH in rat liver of normally-fed state, but 78% of threonine was degraded by TH in liver of 72 h-starved rat and 82% of threonine was metabolized by TH in the liver of rat fed high-protein diet. TA activity was very low in rat liver. We interpret their experimental results as follows: If threonine is metabolized by TH, 2-oxo-butanoic acid is formed. The acid is converted neither to pyurvate nor to methylglyoxal and coverted to propionyl CoA, which can enter in gluconeogenesis. Therefore, threonine plays ketogenic role in liver of normally-fed state, but glucogenic role in liver of rat

starved for 72 h or fed a high protein diet. From the data presented here, it can, therefore, be presumed that threonine is metabolized either by TDH to methylglyoxal or by TA *via* glycine to methylglyoxal in octopus tentacle muscle. Methylglyoxal can be converted to D-lactate, which is in turn converted to pyruvate in the octopus muscle. L-Alanine, cysteine and serine were not utilized for the synthesis of D-lactate, when tested in the same manner as threonine. They might become precursors after an aminotransferase reaction, if they could be incubated with pyruvate of cosubstrate which is good substrate for D-LDH. We measured the contents of glucose, glycerol and L-lactate in the tissue to confirm the data of our previous report. Since the results were similar to those reported previously, they are not shown here. We found, however, that these metabolite levels varied with the nutrition at state of the animal. The results will be reported in the near future.

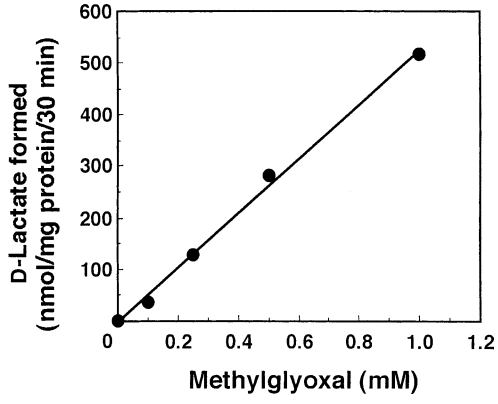

Fig. 4a. D-Lactate formation in the presence of different concentrations of methylglyoxal for 30 min at 25°C in the homogenate of octopus tentacle muscle. The cofactors are shown in Table 1. D-Lactate was converted via pyruvate to 2-methylquinoxalinol which was determined by HPLC

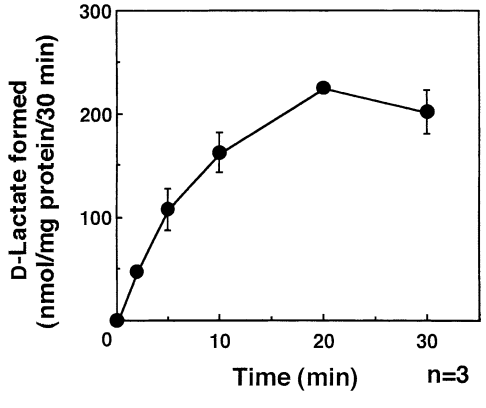

Fig. 4b. Time course of the D-lactate formation from 0.5 mM methylglyoxal at 25°C in 6,000 × *g* supernatant of homogenate of octopus tentacle muscle. D-Lactate was determined as described in Fig. 4a. Results are means ± SE (n = 3)

D-Lactate formation from methylglyoxal

Various amounts of methylglyoxal were added to the dialyzed supernatant of the octopus tentacle homogenate and incubated at 25°C for 30 min. As shown in Fig. 4a, D-lactate formation was linearly dependent upon methylglyoxal concentration. Methylglyoxal (500 μM) was incubated with the supernatant at 25°C and aliquots were taken in regular intervals up to 30 min. Under our assay conditions D-lactate formation increased up to 20 min and after that decreased slightly (Fig. 4b).

Methylglyoxal formation from threonine and glycine

As mentioned above, D-lactate was formed from threonine, glycine (Figs. 1, 2 and 3) and also from methylglyoxal (Fig. 4a) in the octopus tentacle homogenate. These findings indicate that methylglyoxal ought to be

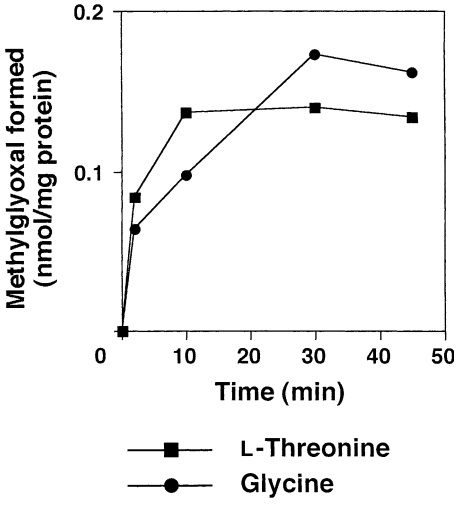

Fig. 5. Time course of methylglyoxal formation from threonine (0.5 mM) and glycine (0.5 mM) at 25°C in the 6,000 × *g* supernatant of octopus tentacle muscle homogenate. The reaction condition are described in Table 1. Methylglyoxal was determined as 2-methylquinoxaline by HPLC

Table 2. Contents of threonine and glycine and enzyme activities relevant to threonine catabolism in octopus tentacle and rat liver

	Octopus tentacle muscle	Rat liver
Threonine*	1.7	0.067 ± 0.04
Glycine*	0.27 ± 0.04	0.24
Threonine dehydrogenase**	0.063 ± 0.018	0.31 ± 0.02
Amine oxidase**	0.045 ± 0.002	not determined

Values are means ± SE, n = 3, *: μmol/g · wet weight, **: mU/mg protein

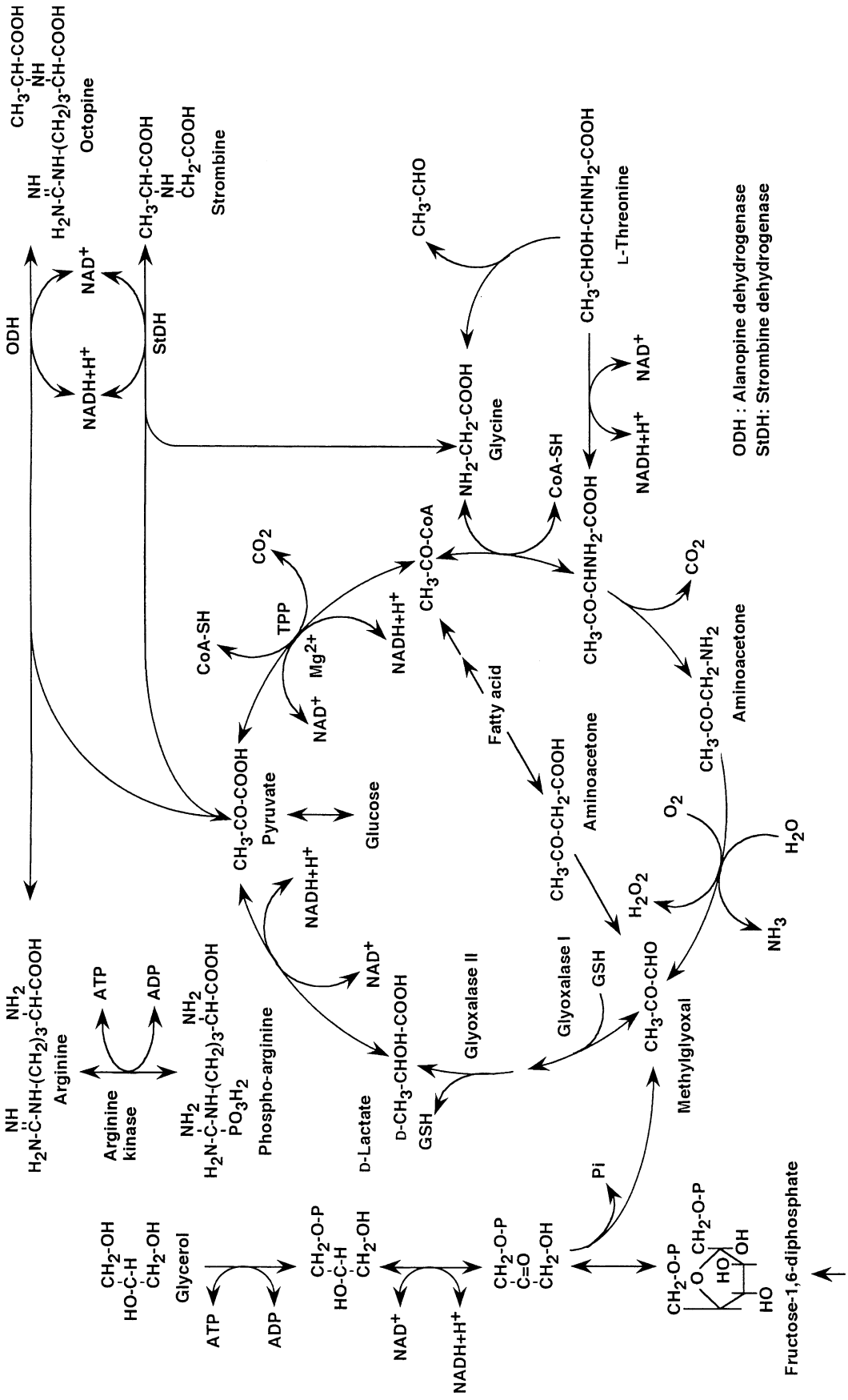

Fig. 6. Proposed pathway for D-lactate metabolism in octopus including threonine and glycine as precursors

formed from threonine and glycine. Threonine and glycine were incubated at 25°C with the dialyzed supernatant of octopus tentacle homogenate. Aliquots were taken in 10 min intervals and the methylglyoxal concentration was determined. The results shown in Fig. 5 indicate that both amino acids were precursors for methylglyoxal.

Presence of threonine and glycine as well as enzyme activities of threonine dehydrogenase and amine oxidase in octopus tentacle muscle

To substantiate the fact that D-lactate is formed from threonine by TDH and TA, it is necessary to prove the presence of amine oxidase activity in octopus tentacle tissue for converting aminoacetone to methylglyoxal. As shown in Table 2, both activities are present in the tissue. The next problems are the contents of threonine and glycine in free form. Threonine concentration was 25 times higher in octopus muscle than in rat liver, and the glycine concentration in both tissue sources was about the same (Table 2). For comparison, Hochachka et al. (1975) reported that threonine and glycine occurs in squid mantle muscle of concentration 6 and 11 μmol per g wet tissue. The latter concentrations are relatively high for physiological intermediates and can be compared to glucose concentration (5 mM) in human blood. Storey and Storey (1978) determined the concentration of threonine and glycine in the muscle of the squid, *Loligo pealeii* as 0.76 and 4.97 μmoles/g wet weight, respectively. These values were at rest state and they did not change after exercise. It is generally said that fresh molluscan animals taste sweet and good because of much contents of glycine. To confirm the presence of the methylglyoxal bypass and the glycolysis pathway in octopus, the activities of phosphofructokinase, pyruvate kinase, aldolase, glycerol kinase, glyoxalase I and II were measured in the octopus (*O. vulgaris*) tentacle muscle. They were similar to the results of our previous report using the same animals (Ohmori et al., 1997). It was confirmed that D-lactate was formed from methylglyoxal and pyruvate. In a conclusion, to summarize and explain the results mentioned above, we present a metabolic map in Fig. 6. As can be seen Fig. 6, threonine can be effectively catabolized as a source of energy. Ammonia formed by this route excreted into water via the ammonotelic path as the final step.

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