A Comparison of Catalytic Properties of Glutamate Dehydrogenase
from Liver and Muscle between Amphibious *Periophthalmus*
cantonensis and Water-breathing Gobid Fishes *Tridentiger*
obscurus obscurus

Katsuya IWATA and Izuru KAKUTA*

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The catalytic properties of glutamate dehydrogenase of the liver and muscle from an amphibious gobid fish, *Periophthalmus cantonensis* were compared with those of the enzymes from a water-breathing gobid fish, *Tridentiger obscurus obscurus*.

The enzyme activities in the liver and muscle of *P. cantonensis* increased to a greater degree with rising ambient salinities than those observed with *T. obscurus obscurus*, when they were acclimated in different salinities for 7 days.

The apparent K_m for NH_4^+ of the muscle enzyme from *P. cantonensis* was about half as much as that of the enzyme from *T. obscurus obscurus*, while those values of the liver enzymes from both species were essentially similar. No species-difference was observed in other kinetic constants of the enzymes from the two gobid fishes including the K_m 's for α -ketoglutarate and NADH, the K_a 's for ADP and leucine and the K_i 's for GTP (in the absence of ADP), NAD^+ and glutamate.

The effect of ADP on the liver enzyme from *P. cantonensis* was stronger than that of the enzyme from *T. obscurus obscurus*, while the effects of ADP on the muscle enzymes from both the species were similar. The effects of ADP on GTP-inhibited enzymes from *P. cantonensis* were much greater than the corresponding effects on the enzymes from *T. obscurus obscurus*. These results suggest that the enzymes from *P. cantonensis* were more tightly regulated by ADP-GTP interaction than the enzymes from *T. obscurus obscurus*.

The mudskipper, *Periophthalmus cantonensis* spends the greatest part of its life on mudflat out of water. Most fish excrete ammonia as the end product of protein metabolism. It has been reported that the level of ammonia excretion by the mudskipper is very low during the period out of water.¹⁻³⁾ Since ammonia is a highly toxic compound, it must be removed, either to the exterior or by conversion into less toxic compounds. It is well known that the lung-fish⁴⁾ as well as semi-terrestrial amphibians⁵⁾ converts ammonia into urea during their terrestrial lives. On the other hand, in the previous work,³⁾ we found that the mudskipper kept either out of water or in a hyper-osmotic medium accumulated a great amount of free amino acids, especially non-essential ones as well as ammonia in its tissue, whereas urea increased to a lesser extent. Under these circumstances, the activities of glutamate dehydrogenase (EC-1.4.1.2.) from liver and muscle markedly increased. In the mudskipper,

therefore, glutamate dehydrogenase catalyzing reductive amination of α -keto-glutarate seems to play a central role in removing ammonia from the tissue while the fish was subjected to conditions of water-shortage.

In the present study, we examined catalytic properties of glutamate dehydrogenase from liver and muscle of *P. cantonensis* kept either out of water or in hyper-osmotic media. In addition, the catalytic properties of *P. cantonensis* were compared with those of a water-breathing gobid fish, *Tridentiger obscurus obscurus* which survives well either in fresh water or in 120% sea water. The present study was undertaken for determining what differences were associated with the transition to an amphibious life-style.

Materials and Methods

P. cantonensis and *T. obscurus obscurus* were collected at the estuary of Tanabe Bay and Susami

* Faculty of education, Wakayama University, Wakayama 640, Japan (岩田勝哉・角田 出: 和歌山大学教育学部生物学教室).

River in Wakayama Prefecture respectively. They were kept in 20% sea water (S.W.) for a month before use. Throughout the rearing periods, chironomid larvae were daily supplied as a food. The mudskipper was then acclimated in 20, 50 and 80% S.W. for 7 days, and kept out of water for 3 days as described previously.⁹⁾ *T. obscurus obscurus* was directly transferred to fresh water, 20, 50, 80 and 120% S.W., and acclimated in each medium for 7 days. During the period of acclimation, both species were starved and held at a temperature of $20^{\circ}\text{C} \pm 1$.

Liver and muscle tissues from both species were dissected out, blotted, and homogenized in 5 vol. of 0.25 M sucrose with 0.1 mM EDTA in 3 mM Tris-HCl buffer (pH 7.4). No purification of the enzyme was attempted, and the crude mitochondrial fraction was used for the enzyme assay. The procedures for the preparation of mitochondrial fraction and for the enzyme assay were the same as previously described.⁹⁾ For purposes of comparison, the enzyme activities in gill and skin from both species were also examined. The enzyme activity was expressed as specific activity-unit ($\mu\text{mol NADH}/\text{min.}/\text{mg protein}$).

The apparent K_m values for substrates were determined by means of Lineweaver-Burk plots using at least seven different concentrations of substrate. The apparent K_a and K_i values were estimated from Lineweaver-Burk plots and Dixon plots respectively. Kinetic constants for allosteric effectors were derived from Hill plots. Standard deviations were calculated for enzyme activities and for kinetic constants. Results were compared using Student's *t*-test.

Results

Effect of pH

The pH dependence of the liver enzyme from *P. cantonensis* kept out of water is shown in Fig. 1. The pH optimum in the direction of reductive amination was about 7.5, in the reverse direction it was 8.5–9.0.

The activity of the liver enzyme as assayed in the direction of oxidative deamination was very low, which was about 10% of that in the reductive amination in the presence of ADP. In common with glutamate dehydrogenase from other fish sources,^{8–10)} the liver enzyme showed very low activity with NADPH as coenzyme. The activity with NADP^+ could not be detected in the present assay condition.

Fig. 1. The pH profiles of glutamate dehydrogenase from the liver of *P. cantonensis*. Enzyme was assayed in both the directions of the reductive amination (NADH and NADPH) and of the oxidative deamination (NAD^+) under the optimal conditions (in the presence of 0.15 mM ADP).

The enzyme from the muscle of *P. cantonensis* as well as the enzymes from the liver and muscle of *T. obscurus obscurus* was found to be similar in these respects.

Enzyme Activities

The activities of the enzymes from liver, muscle, skin and gill from both species as assayed in the reductive amination using the optimal conditions are shown in Fig. 2. Of the tissues examined, the liver of both species had the highest enzyme activities, and the muscles showed the lowest. In each tissue from both species, the enzyme activity increased with rising ambient salinities. The activity in each tissue from *T. obscurus obscurus* increased a lesser degree with rising salinity as compared with that from *P. cantonensis*, although the activity in each tissue, especially in the liver from *T. obscurus obscurus* kept in 20% S.W. (Control) was much higher than that of *P. cantonensis*. The activity in each tissue from *T. obscurus obscurus* kept in 120% S.W. was 1.3- to 1.8-fold of that in 20% S.W., while the activity of *P. cantonensis* kept in 80% S.W. increased 2- to 3-fold of that in 20% S.W. In *P. cantonensis* kept out of water, the enzyme activity in each tissue increased 3- to 4-fold of that in 20% S.W.

Fig. 2. The activities of glutamate dehydrogenase from various tissues of *P. cantonensis* (Left) and of *T. obscurus obscurus* (Right) kept either in different salinities or out of water. Enzymes were assayed in the direction of reductive amination under the optimal conditions (in the presence of 0.15 mM ADP). The bar on each point indicates the standard deviation. Liver (⊕—⊕), Gill (●—●), Skin (○—○), Muscle (●—●).

Table 1. Apparent K_m values of glutamate dehydrogenase from liver and muscle of *P. cantonensis* and of *T. obscurus obscurus*

Kinetic constants, mM	<i>P. cantonensis</i>		<i>T. obscurus obscurus</i>	
	Liver	Muscle	Liver	Muscle
K_m Ammonia (—ADP)	86.24±6.59 (16)	59.73±5.50 (16)	116.04±19.09 (6)	100.00±25.49 (6)
K_m Ammonia (+ADP)	66.08±7.81 (8)	40.05±6.57 (8)	85.80±10.73 (6)	83.72±10.97 (6)
K_m α -ketoglutarate (—ADP)	0.64±0.10 (3)	0.56±0.02 (3)	0.63±0.01 (3)	0.85±0.02 (3)
K_m α -ketoglutarate (+ADP)	1.16±0.21 (3)	1.05±0.10 (3)	1.25±0.04 (3)	1.10±0.06 (3)
K_m NADH (—ADP)	0.018±0.003 (3)	0.021±0.006 (3)	0.017±0.002 (3)	0.015±0.001 (3)
K_m NADH (+ADP)	0.032±0.004 (3)	0.031±0.004 (3)	0.046±0.002 (3)	0.038±0.001 (3)

When added, ADP concentration was 0.15 mM. Figures in parentheses indicate the number of sample

(Mean±S.D.)

Kinetic Constants

The apparent K_m values for NH_4^+ , α -ketoglutarate and NADH are listed in Table 1. The enzymes from both species showed kinetics of simple Michaelis type for these substrates. The apparent K_m values for NH_4^+ of the liver enzyme from *P. cantonensis* in the absence and the presence of ADP (0.15 mM) were higher than those values observed with the muscle enzyme ($p < 0.001$). The present K_m values for NH_4^+ of the enzymes from *P. cantonensis* were slightly different from those of the previous report⁹ which were estimated using insufficient number of

samples.

There was no difference between the K_m 's for NH_4^+ of the liver enzyme and those of the muscle enzyme from *T. obscurus obscurus*. It should be noted that the K_m 's for NH_4^+ of the muscle enzyme from *T. obscurus obscurus* in either the absence or the presence of ADP were about twice as much as those observed with the enzyme from *P. cantonensis*. The K_m 's for NH_4^+ of the enzymes from gill and skin of both species were similar to those of the liver enzyme either from *P. cantonensis* or from *T. obscurus obscurus*. The enzymes from both species had essentially similar K_m

values for α -ketoglutarate in either the absence or the presence of ADP. No difference was also found in the apparent K_m values for NADH of the enzymes from the two gobiid fishes.

The apparent K_m values of the liver and muscle enzymes from both species for substrates as mentioned above were not different among the fishes adapted to different conditions.

Activators and Inhibitors

The effects of activators and inhibitors on the enzymes are shown in Table 2. Of the activators examined, ADP was the most effective, and the liver enzyme from *P. cantonensis* was activated to a greater degree by ADP than that from *T. obscurus obscurus*, while the effects of ADP on the muscle enzymes from both species were similar. The curve relating the enzyme activity to ADP concentration was sigmoidal for the liver and

muscle enzymes from both species. This curve suggests an allosteric interaction between the enzymes and ADP. In accordance with the enzymes from non-mammalian sources,^{9,10} no activation by ADP was observed in the enzymes from both species when NADPH was used as coenzyme. The effects of ATP, AMP and leucine on the liver enzymes from both species were similar to the corresponding effects on the muscle enzymes. The activation curves of these activators were hyperbolic for the enzymes from both species.

Once the enzymes from both species were fully activated by ADP, they were not further stimulated by the addition of other activators, whereas the enzymes partially activated by ATP, AMP, leucine and inorganic salts were further activated to a maximum activity by the addition of ADP. The enzymes of both tissues either from *P. cantonensis*

Table 2. Effects of activators, inhibitors and their interaction with ADP (relative activities)

	<i>P. cantonensis</i>				<i>T. obscurus obscurus</i>			
	Liver		Muscle		Liver		Muscle	
	-ADP	+ADP	-ADP	+ADP	-ADP	+ADP	-ADP	+ADP
No addition	100%	600%	100%	350%	100%	380%	100%	320%
Activator								
ATP (1.5 mM)	156	542	154	336	174	370	150	315
AMP (1.0 mM)	192	563	189	326	174	374	164	305
Leucine (5 mM)	239	537	249	354	271	360	224	300
MgCl ₂ (100 mM)	187	—	192	—	174	—	185	—
NaCl (200 mM)	138	600	146	350	—	—	—	—
KCl (100 mM)	132	—	145	—	—	—	—	—
MgSO ₄ (50 mM)	117	—	113	—	—	—	—	—
Na ₂ SO ₄ (50 mM)	129	—	143	—	—	—	—	—
Inhibitor								
GTP (0.01 mM)	19	468	19	263	13	127	16	85
NAD ⁺ (2 mM)	39	295	31	134	49	243	44	150
Glutamate (32 mM)	53	338	50	190	50	240	55	138
Succinate (5 mM)	70	472	84	302	63	254	79	243

Enzymes were assayed in the direction of reductive amination under the optimal conditions. When added, ADP concentration was 0.15 mM.

Table 3. Apparent K_a and K_i values of glutamate dehydrogenase from liver and muscle of *P. cantonensis* and of *T. obscurus obscurus*

Kinetic constants, mM	<i>P. cantonensis</i>		<i>T. obscurus obscurus</i>	
	Liver	Muscle	Liver	Muscle
K_a ADP	0.023 ± 0.002 (16)	0.031 ± 0.002 (16)	0.024 ± 0.001 (3)	0.026 ± 0.002 (3)
K_a Leucine (-ADP)	0.098 ± 0.010 (3)	0.116 ± 0.010 (3)	0.098 ± 0.012 (3)	0.087 ± 0.016 (3)
K_i GTP (-ADP)	0.0013 ± 0.0001 (7)	0.0013 ± 0.0001 (7)	0.0012 ± 0.0001 (3)	0.0013 ± 0.0001 (3)
K_i GTP (+ADP)	0.0201 ± 0.0017 (8)	0.0245 ± 0.0020 (8)	0.0030 ± 0.0004 (3)	0.0050 ± 0.0007 (3)
K_i NAD ⁺ (+ADP)	0.87 ± 0.18 (3)	0.79 ± 0.24 (3)	0.65 ± 0.04 (3)	0.69 ± 0.13 (3)
K_i Glutamate (+ADP)	15.16 ± 1.72 (5)	11.33 ± 1.45 (5)	14.52 ± 0.80 (3)	17.29 ± 1.63 (3)

The condition of enzyme assay was the same as in Table 2.

(Mean $\pm$ S.D.)

or from *T. obscurus obscurus* had essentially similar K_a values for ADP (Table 3). No difference was also found among the apparent K_a values for leucine.

Of several inorganic salts examined, $MgCl_2$ was the most effective in activating the enzymes from *P. cantonensis* and $MgSO_4$ was the lowest. On the other hand, the effect of Na_2SO_4 on the enzymes was similar to that of NaCl as well as KCl. The effect of $MgCl_2$ on the enzymes from *T. obscurus obscurus* was similar to that of the enzymes from *P. cantonensis*.

In the direction of reductive amination, the enzymes from both species were inhibited by the reaction products, NAD^+ and glutamate. Not any species-difference was found in the effects of NAD^+ or of glutamate on the enzymes examined. Similarly, no difference was observed in the effects of succinate on the enzymes from the two gobiid fishes. The greatest degree of inhibition was obtained with GTP, and GTP at a concentration of 0.01 mM reduced the activities of the enzymes from both species to less than 20% of the activities in the absence of GTP. The subsequent addition of ADP (0.15 mM) to GTP-inhibited enzymes restored the activities. This effect of ADP was more pronounced for the enzymes of both tissues from *P. cantonensis* than that for the enzymes from *T. obscurus obscurus*; ADP restored about 80% of the activities in the absence of GTP for the enzymes from *P. cantonensis* as compared with 30% for the enzymes from *T. obscurus obscurus*. On the other hand, for the succinate-inhibited enzymes, a degree of releasing the inhibition by ADP was not different among the enzymes examined (70–90% of the activities was restored). For the liver and muscle enzymes from both species inhibited by reaction products, ADP was less effective in releasing the inhibition, restoring 40–60% of the activities.

A clear species-difference was not observed in either the apparent K_i values for NAD^+ or the K_i 's for glutamate of the enzymes examined. No difference was also observed among the K_i 's for GTP in the absence of ADP (Table 3). On the other hand, in the presence of ADP, the apparent K_i values for GTP of the enzymes from *P. cantonensis* were much higher than those observed with the enzymes from *T. obscurus obscurus*; ADP increased the K_i values of the enzymes from *P. cantonensis* 15- to 20-fold compared with 3- to 4-fold of the enzymes from *T. obscurus obscurus*.

Discussion

The liver and muscle enzymes from the two gobiid fishes resemble the enzymes from other non-mammalian sources in several aspects including the apparent K_m for substrate (NH_4^+ , α -ketoglutarate and NADH), activation by ADP, AMP and leucine, inhibition by GTP, succinate and reaction products (NAD^+ and glutamate) and a lower specificity for NADPH as coenzyme.^{8–11} It should be noted that the enzymes from *P. cantonensis*, especially the liver enzyme, were more strongly affected by ADP-GTP interaction than those from *T. obscurus obscurus* as well as the enzyme from the eel liver.¹⁰ This fact suggests that the enzymes from *P. cantonensis* are more tightly regulated by the energy status of the cell. A similar difference is also observed between glutamate dehydrogenase from kidneys of air- and water-breathing osteoglossiform fishes.⁹

Although little information is available about the properties of the muscle enzyme from other fish sources, the catalytic properties of the muscle enzyme from *P. cantonensis* were different from those of the muscle enzyme from *T. obscurus obscurus* as well as the liver enzymes from both species. In particular, it is interesting to note that of the tissues from both species examined, the muscle enzyme from *P. cantonensis* had the lowest K_m value for NH_4^+ . In general, the K_m of glutamate dehydrogenase for NH_4^+ is high as compared with the concentration *in vivo*, and the glutamate dehydrogenase from fish sources is considered to play an important role for the oxidative deamination rather than the reductive amination under normal circumstances.^{12–14} However, in the case of *P. cantonensis* kept out of water, the ammonia level in the muscle increased up to about 20 mM/kg tissue water, and in accordance with this, free amino acids, especially non-essential ones increased greatly.⁹ In addition, TAMURA *et al.*¹⁵ reported that oxygen consumption of *P. cantonensis* kept out of water decreased about 60% of that kept in a diluted sea water. This finding implies that the tissue, especially the muscle of *P. cantonensis* is exposed to a hypoxic condition during the period out of water. Any increase in NADH and ADP as a result of hypoxic stress would strongly activate the reductive amination of α -ketoglutarate. Thus, the glutamate dehydrogenase from the muscle of *P. cantonensis* seems to play an important role in

removing ammonia from the tissue by means of reductive amination of α -ketoglutarate, otherwise ammonia may reach a toxic level. Since the activities of the liver enzyme from *P. cantonensis* increased greatly when the fish was placed in conditions of water-shortage, the liver enzyme would also play an important role for the regulation of amino acid pool in the whole body, functioning either direction of amination or deamination depending on a physiological state of the fish.

It has been known that in *T. obscurus obscurus* (unpublished) as well as several euryhaline teleosts adapted to a hyper-osmotic medium, free amino acids were accumulated greatly in their muscle tissues, which play an important role in cell volume regulation.¹⁶⁻¹⁸⁾ Therefore, the function of the muscle enzyme catalyzing the reductive amination as in *P. cantonensis* appears to be rather general in euryhaline teleosts. However, a lower *K_m* value for NH_4^+ of the muscle enzyme from *P. cantonensis* together with a strong ADP-GTP interaction appears to be well suited for amphibious mode of life. Thus, in conclusion, the glutamate dehydrogenase of *P. cantonensis*, especially the muscle enzyme appears to have been modified to perform a primarily reductive amination in keeping with a need of detoxication of ammonia as well as of cell volume regulation during its terrestrial life.

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