Purification and Properties of Dolphin Muscle Aspartate and Alanine Transaminases and their Possible Roles in the Energy Metabolism of Diving Mammals

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(Received 29 May 1974)

1. Mitochondrial and supernatant aspartate transaminases (EC 2.6.1.1) and supernatant alanine transaminase (EC 2.6.1.2) were purified 89-, 204- and 240-fold respectively, from dolphin muscle. Starch-gel electrophoresis of crude and purified preparations revealed that all three enzymes exist as single forms. 2. K_m values of α -oxoglutarate, alanine, pyruvate and glutamate for the alanine transaminase were 0.45, 8.2, 0.87 and 15 mM respectively. For the aspartate transaminases, the K_m values of α -oxoglutarate, aspartate, oxalacetate and glutamate were 0.76, 0.50, 0.10 and 9.4 mM respectively, for the mitochondrial form and 0.13, 2.4, 0.06 and 3.2 mM respectively, for the supernatant form. 3. In all cases, as the assay pH value was decreased from pH 7.3, the K_m values of the α -oxo acids decreased whereas those of the amino acids increased. 4. The apparent equilibrium constants for the aspartate transaminases were independent of pH. These values were 9.2 and 6.8 for the mitochondrial and supernatant forms respectively, where $K'_{\text{equl.}} = \frac{[\text{aspartate}][\alpha\text{-oxoglutarate}]}{[\text{glutamate}][\text{oxaloacetate}]}$. 5. Studies of the inhibition of the aspartate transaminases by dicarboxylic acids indicated that these enzymes may be controlled by pools of metabolic intermediates. 6. Three key roles are suggested for the transaminases in the energy metabolism of the diving animal. First, it is believed that a combined action of the transaminases could enhance energy production during hypoxia by providing (a) fumarate from aspartate for the ATP-producing reversal of succinate dehydrogenase, and (b) α -oxoglutarate from glutamate for the GTP-producing succinyl thiokinase reaction. Secondly, diving mammals probably accumulate more NADH than other mammals during hypoxia. The aspartate transaminases seem particularly well suited for restoring and maintaining redox balance via the malate–aspartate cycle after aerobic metabolism is resumed. Finally, since the preferred fuel for aerobic work is fat, the combined reactions of the transaminases could be instrumental in providing increased supplies of oxaloacetate for sparking the tricarboxylic acid cycle.

Despite impressive physiological adaptations for O_2 storage and conservation in diving mammals, O_2 in the muscle may be totally consumed long before the end of the dive and subsequent muscle work must be sustained by ATP produced anaerobically (Andersen, 1966; Ewer, 1947; Scholander, 1940, 1962). Although all aspects of this anaerobiosis have not been investigated, the conversion of glucose into lactate via glycolysis is considered to be a major source of ATP during prolonged diving (George *et al.*, 1971; Kerem *et al.*, 1973; Reeves, 1963).

This pathway can be rapidly stimulated in the dive to produce significant ATP supplies in the complete absence of O_2 , but the accumulation of lactate causes acidification which results in muscle fatigue and the energy yield, moles of ATP/mol of

glucose, is low. It therefore seemed reasonable to question whether or not other ATP-producing reactions could function during anaerobiosis in diving mammals particularly since, in invertebrates, altered glycolytic pathways have been elucidated which couple additional phosphorylations to those of normal glycolysis (Barrett & Beis, 1973; Hochachka & Mustafa, 1972; Saz, 1971; DeZoeten & Tipker, 1969; DeZwaan & Marrewijk, 1973). In these invertebrates, one of the traits of their altered anaerobic metabolism is the accumulation of anaerobic end-products other than lactate, such as succinate, alanine and propionate.

Since succinate has been observed to accumulate in a variety of hypoxic mammalian tissues (Hoberman & Prosky, 1967; Kondrashova & Chahovets, 1971; Mensen de Silva & Cazorla, 1973; Weil-Malherbe, 1937) and succinate and alanine accumulate in the blood of diving vertebrates after a dive (Hochachka

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et al., 1974), it becomes increasingly attractive to suggest that anaerobic ATP-producing pathways similar to those found in the invertebrates do indeed occur in diving mammals. This idea is further supported by the observation that mitochondrial fumarate reduction and α -oxoglutarate oxidation to succinate both produce ATP in hypoxic mammalian tissues (Chance & Hollunger, 1960; Hunter, 1949; Penney & Cascarano, 1970; Randall & Cohen, 1966; Sanadi & Fluharty, 1963; Wilson & Cascarano, 1970), as seen in the invertebrates (see Hochachka & Mustafa, 1972). In the invertebrates, the source of fumarate appears to be phosphoenolpyruvate-derived oxaloacetate but this source is unlikely in the mammals since the enzyme responsible for this conversion, phosphoenolpyruvate carboxykinase, favours phosphoenolpyruvate formation in mammalian tissues (Saz, 1971). We therefore considered that the transamination of free aspartate via aspartate transaminase and of glutamate via alanine transaminase could provide fumarate and α -oxoglutarate respectively, for these anaerobic phosphorylations in the muscle of diving mammals. This idea is consistent with data (Safer & Williamson, 1973; Safer *et al.*, 1971) suggesting that augmentation of tricarboxylic acid-cycle intermediates can occur at the expense of free amino acids through the combined action of these two transaminases. We therefore purified and kinetically characterized both enzymes to determine their role in the energy metabolism of diving mammals with particular reference to the possibility that they may provide substrates for ATP-producing reactions during anaerobiosis.

Materials and Methods

Reagents

All substrates, cofactors, commercial enzymes and metabolites used in inhibition experiments were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); starch was from Connaught Research Laboratories (Toronto, Ont., Canada); reagents for polyacrylamide-gel electrophoreses were from Eastman Organic Chemicals Dept. (Rochester, N.Y., U.S.A.); CM-cellulose and DEAE-cellulose were from Whatman Biochemicals Inc. (Clifton, N.J., U.S.A.); and Sephadex G-200 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of reagent grade. The dolphin muscle (*Lagenorhynchus obliquidens*) was generously donated by the Vancouver Public Aquarium, Vancouver, B.C., Canada and stored at -20°C .

Methods

Enzyme assays. All assays contained NADH at a concentration of 0.2 mM, unless otherwise indicated, and were adjusted to a final volume of 2.5 ml with 100 mM-sodium phosphate buffer. Assays were

started by the addition of 0.1 ml of enzyme preparation containing approx. 3 units/ml. The $\Delta E_{340}/\text{min}$ was then determined at 37°C by using a Unicam SP.800 or Unicam Pye SP.1800 spectrophotometer. This is referred to as the 'standard method'.

A survey of transaminases was made by using duplicate non-coupled assays containing 15 mM- α -oxo acid, 30 mM-glutamate and 0.1 ml of enzyme preparation (dolphin muscle homogenized in 4 vol. of assay buffer, pH 7.3, and centrifuged at 600g for 15 min) in a final volume of 2.0 ml adjusted to pH 7.3. A control assay containing no glutamate was prepared for each α -oxo acid. Two additional control assays contained no α -oxo acid. Portions (0.6 ml) of the reaction mixtures were added to 0.6 ml of 10% (w/v) trichloroacetic acid at timed intervals. The precipitate was sedimented and a 0.5 ml sample of the supernatant was transferred to a cuvette, neutralized with 1 M-NaOH and assayed for α -oxoglutarate content by the standard method with 0.02 ml of glutamate dehydrogenase [200 units/ml in 2.0 M-(NH_4) $_2\text{SO}_4$].

Glutamate dehydrogenase was assayed by the standard method at pH 7.3 with 2 mM- α -oxoglutarate and 2 mM- NH_4Cl .

All alanine and aspartate transaminase assays, unless otherwise indicated, were by the standard method. Alanine transaminase assays in the direction of glutamate production contained various amounts of α -oxoglutarate and alanine and 0.02 ml of lactate dehydrogenase [650 units/ml in 0.21 M-(NH_4) $_2\text{SO}_4$]. Aspartate transaminase assays in the direction of glutamate production contained various amounts of α -oxoglutarate and aspartate and 0.02 ml of malate dehydrogenase [500 units/ml in 0.28 M-(NH_4) $_2\text{SO}_4$]. Alanine and aspartate transaminase assays in the direction of α -oxoglutarate production contained various amounts of pyruvate and oxaloacetate respectively, various amounts of glutamate and 0.02 ml of glutamate dehydrogenase [300 units/ml in 2.0 M-(NH_4) $_2\text{SO}_4$]. No activity was observed with any of the transaminase preparations used for kinetic studies when either the α -keto acid or amino acid was omitted from any of the assay mixtures. The presence of excess of coupling enzyme was confirmed by adding either oxaloacetate, or pyruvate or α -oxoglutarate to representative assays after the assay had progressed for 60–90 s. Appropriate corrections were made to account for the α -oxoglutarate introduced into the aspartate transaminase assays via the enzyme preparations which contained 2 mM- α -oxoglutarate for stabilization of the enzyme. All substrate and inhibitor stock solutions were adjusted to the pH values of the assays. All assays were conducted at pH 7.3 ('normal') since this was the optimum pH value for both transaminases and at pH 6.8 or 6.3 ('hypoxic') since pH decreases as hypoxia progresses (Chance *et al.*, 1964).

Lactate and malate dehydrogenases were assayed by the standard method at pH 7.3 and 1 mM-pyruvate and -oxaloacetate respectively.

Enzyme units. These are defined as μmol of product formed/min at pH 7.3 and 37°C. Aspartate and alanine transaminase specific activities were measured at 10 mM-aspartate and -alanine respectively, and 5 mM- α -oxoglutarate.

Aspartate transaminase specificity. The α -keto acids of tyrosine, phenylalanine and leucine were substituted for oxaloacetate to a final concentration of 1 mM in the standard aspartate transaminase assay in the direction of α -oxoglutarate production at pH 7.3. The rates of reduction of the α -oxo acids by NADH were determined in the absence of glutamate and subtracted from the activities obtained after the transaminase reactions were started by the addition of glutamate to a concentration of 20 mM. To ascertain that the α -oxo acids were not inhibiting the coupling enzyme, α -oxoglutarate was added after the assays had progressed for 3–5 min.

Protein determinations. Protein determinations were calculated by using the formula

$$(E_{215}^{1\text{cm}} - E_{225}^{1\text{cm}}) \times 0.154 = \text{mg of protein/ml}$$

(Murphy & Kies, 1960).

Electrophoresis. Horizontal starch gels (13%, w/v) were run at pH 7.0 and 250 V for 18–24 h at 4°C with 135 mM-Tris–43 mM-citrate buffer (Shaw & Prasad, 1970) in the tanks and a 1:14 dilution of the same buffer being used for the gels. The gels were sliced in half lengthwise before being stained.

Polyacrylamide-gel electrophoresis was conducted by Davis' (1964) method at 4°C by using 7% (w/v) separating gels, a Büchler apparatus and 1% (w/v) Amido Schwartz in 7% (w/v) acetic acid to stain for protein.

The stain for aspartate transaminase consisted of aspartate (500 mg), α -oxoglutarate (75 mg), pyridoxal 5-phosphate (50 mg) and Fast Violet B salt (200 mg) in 100 ml of 50 mM-Tris–HCl buffer adjusted to a final pH of 7.6.

The stain for alanine transaminase consisted of alanine (250 mg), NADH (15 mg) and α -oxoglutarate (37.5 mg) adjusted to pH 7.3 in 50 ml of 50 mM-Tris–HCl buffer (solution A). Difco Bactoagar (1 g; Canlab, Vancouver, B.C., Canada) was boiled in another 50 ml of the same buffer until it dissolved (solution B). Both solutions were brought to 42°C and combined after 750 units of lactate dehydrogenase had been added to solution A. This mixture was poured over the surface of the sliced starch gel. After approximately 30 min at room temperature the solidified agar gel was peeled from the surface of the starch gel and the alanine transaminase could be localized by viewing the gel under a u.v. lamp equipped with a 340 nm filter.

Results

Enzyme specific activities

The α -oxo acids of aspartate, alanine and tyrosine were transaminated at relative rates of 100, 12 and 0.3 per g of tissue respectively by crude dolphin muscle preparations in the non-coupled transaminase assay. There was no measurable activity with the α -oxo acids of cysteine, glycine, isoleucine, phenylalanine or valine; nor was there measurable glutamate dehydrogenase activity in agreement with the findings of Copenhaver *et al.* (1950).

Purification of alanine transaminase

All procedures were conducted at 4°C unless otherwise indicated.

Step 1: dolphin muscle (300 g) was homogenized in 900 ml of buffer A (50 mM-Tris–HCl–10 mM-EDTA–10 mM-2-mercaptoethanol, pH 7.0 at 25°C) in a Sorvall Omnimixer at full speed for 60 s and centrifuged at 10000g for 40 min. The supernatant (775 ml) was placed in a 60°C water bath, brought to 52°C and immediately placed in an ice-bath until its temperature was 4°C.

Step 2: the heat-treated sample (775 ml) was brought to 30% (w/v) saturation with 136 g of $(\text{NH}_4)_2\text{SO}_4$, stirred for 30 min and centrifuged at 10000g for 30 min. The supernatant (790 ml) was brought to 55% (w/v) saturation with 128 g of $(\text{NH}_4)_2\text{SO}_4$, stirred for 30 min and centrifuged at 10000g for 30 min. The pellet was redissolved in a minimum amount of buffer B (15 mM-Tris–HCl–2 mM-EDTA–10 mM-2-mercaptoethanol, pH 7.25 at 25°C) and centrifuged at 39000g for 30 min.

Step 3: the centrifuged sample was placed on a column (4 cm $\times$ 60 cm) of Sephadex G-200 equilibrated with buffer B. The column was eluted at 17 ml/h and 4 ml fractions were collected. The peak fractions were pooled and adjusted to the same pH value and conductivity as buffer B.

Step 4: the sample was applied to a column (1.9 cm $\times$ 25 cm) of DEAE-cellulose equilibrated to buffer B. The column was flushed with 600 ml of buffer B to free the column of all lactate dehydrogenase activity and the alanine transaminase then removed by applying a linear gradient of 0–100 mM-NaCl in buffer B (300 ml reservoirs). The column was eluted at 30 ml/h and 3 ml fractions were collected. The peak fractions were pooled, taken to 20 mM-EDTA for stabilization (Saier & Jenkins, 1967) and stored at 4°C. Unlike the alanine transaminase purified from pig heart by Saier & Jenkins (1967), the dolphin muscle enzyme was totally inactivated after one freeze–thaw cycle. However, when stored at 4°C, the purified preparations were stable for periods of at least 2 months. A typical purification procedure is summarized in

Table 1. *Purification of dolphin muscle alanine transaminase*

For details of fractionation see the text.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold- purification	Yield (%)
Crude	1750	11700	0.15	1.0	100
Heat-treated	1700	9750	0.18	1.2	98
30–55% (w/v) (NH ₄) ₂ SO ₄	1400	1180	1.2	8.0	80
Sephadex G-200	1000	260	3.9	26	57
DEAE-cellulose	606	17	35.5	237	35

Table 1. The highest specific activity obtained was 36.8 units/mg of protein and all preparations were totally free of lactate dehydrogenase activity.

Purification of aspartate transaminase

Initial attempts at purification of aspartate transaminase failed owing to instability of the enzyme. EDTA, 2-mercaptoethanol, dithiothreitol, glutamate, aspartate, pyridoxal 5-phosphate and α -oxoglutarate were tested as stabilizers against heat denaturation for 30 min at 75°C. A mixture of pyridoxal 5-phosphate and α -oxoglutarate were the most effective stabilizers and were included in all buffers used for purification and storage of the enzyme. All steps of the purification were conducted at 4°C unless otherwise indicated.

Step 1: dolphin muscle (136g) was homogenized in 408 ml of buffer C (100 mM-sodium phosphate–2 mM- α -oxoglutarate–0.25 mM-pyridoxal 5-phosphate, pH 7.3) in a Sorvall Omnimixer at full speed for 60 s and centrifuged at 10000g for 30 min. The dolphin muscle pellets took up large quantities of buffer and always weighed more than the original portion of tissue. Consequently the pellet was rehomogenized in 1 vol. of buffer and centrifuged. This process was repeated again and the supernatants from each step were pooled. This rehomogenization procedure significantly increased the yield of aspartate transaminase to 135 units/g of tissue. Alanine transaminase activity was also measured and was found to be 16 units/g of tissue.

Step 2: the pooled supernatants (630 ml) were placed in a 60°C water bath for 40 min with frequent mixing and then centrifuged at 10000g for 30 min.

Step 3: the supernatant (570 ml) was taken to 50% (w/v) saturation with 178 g of (NH₄)₂SO₄, stirred for 30 min and centrifuged at 12000g for 30 min. The pellet was discarded and the supernatant (640 ml) was taken to 75% (w/v) saturation with 113 g of (NH₄)₂SO₄, stirred for 30 min and centrifuged at 12000g for 40 min. The pellet was redissolved in buffer C to a final volume of 24 ml and dialysed against 4 litres of buffer D (15 mM-sodium phosphate–2 mM- α -oxoglutarate–0.25 mM-pyridoxal

5-phosphate, pH 7.0) for 8 h. The contents of the dialysis bag were concentrated by covering the bag with crystalline sucrose for 11 h. The contents were then forced down to one end of the dialysis bag and the bag was tied off and dialysed against 4 litres of buffer D for 4 h.

Step 4: the contents of the dialysis bag (16 ml) were centrifuged at 30000g for 30 min and then applied to a column (5 cm × 100 cm) of Sephadex G-200 equilibrated with buffer D plus 0.02% sodium azide (to prevent bacterial growth). The column was eluted at 20 ml/h and 5 ml fractions were collected after 900 ml of buffer had passed through the column. The peak fractions were pooled and adjusted to the same pH value and conductivity as buffer E (10 mM-citrate–sodium phosphate–2 mM- α -oxoglutarate–0.25 mM-pyridoxal 5-phosphate–0.02% sodium azide, pH 6.5).

Step 5: the sample (197 ml) was applied to a column (1.6 cm × 10 cm) of CM-cellulose equilibrated to buffer E. The column was washed with 50 ml of buffer E. The material that passed through the column contained all of the supernatant (anionic) aspartate transaminase whereas all of the mitochondrial (cationic) isoenzyme remained on the column. The separation of the two forms at this step was complete by the criterion of starch-gel electrophoresis. A linear gradient of 0–100 mM-NaCl in buffer E (200 ml reservoirs) was applied at 30 ml/h and 3 ml fractions were collected. The peak fractions containing the aspartate transaminase were pooled, taken to 90% (w/v) saturation with (NH₄)₂SO₄ and centrifuged at 30000g for 30 min. The pellet was dissolved in buffer D.

Step 6: the material that passed through the CM-cellulose column and contained all of the supernatant isoenzyme was taken to 80% (w/v) saturation with (NH₄)₂SO₄ and centrifuged at 30000g for 30 min. The pellet was redissolved in buffer F (5 mM-Tris–HCl–0.75 mM- α -oxoglutarate–0.10 mM-pyridoxal 5-phosphate, pH 8.0 at 25°C) and dialysed against 1 litre and 2 litres of buffer F for 2 and 18 h respectively. The sample was centrifuged at 30000g for 30 min, adjusted to the same pH value and conductivity as buffer F and applied to a column (1.6 cm × 10 cm) of DEAE-

Table 2. *Purification of dolphin muscle aspartate transaminase*

For details of fractionation see the text.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold- purification	Yield (%)
Crude	18270	16384	1.12	1.0	100
Heat-treated	18896	6755	2.80	2.5	103
50–75% (w/v) (NH ₄) ₂ SO ₄	16944	922	18.4	16	93
Sephadex G-200	12327	349	35.4	32	67
CM-cellulose (mitochondrial isoenzyme)	5200	22.8	228	204	28
DEAE-cellulose (supernatant isoenzyme)	1880	56.9	33.0	29	10
Electrofocused (supernatant isoenzyme)	4.34/ml	0.0432/ml	100	89	—

cellulose equilibrated with buffer F. The column was washed with 40ml of buffer F and then a gradient of 0–200mm-NaCl in buffer F (300ml reservoirs) was applied at 30ml/hr and 3ml fractions were collected. The peak fractions were pooled, taken to 80% (w/v) saturation with (NH₄)₂SO₄ and centrifuged at 30000g for 30min. The pellet was redissolved in buffer D.

Step 7: Both isoenzyme preparations were dialysed against 2 litres of buffer D plus 0.02% sodium azide for 18h and then centrifuged at 30000g for 30min. Both forms were stable at 4°C for at least 4 months when redialysed against buffer D plus azide every 6 weeks to replenish the α -oxoglutarate and the pyridoxal 5-phosphate. The mitochondrial transaminase preparation contained no malate dehydrogenase contamination but the supernatant enzyme preparation did contain malate dehydrogenase activity that had co-purified with the supernatant transaminase. This preparation was used for all of the experiments except for the determination of kinetic constants with oxaloacetate and glutamate as substrates. For these determinations, further purification was necessary.

Step 8: supernatant enzyme preparation (1ml) was electrofocused with the anode at the top of a 110ml L.K.B. electrofocus column by using a pH gradient of 5–8. The voltage was increased 50V every 15min from 300 to 700V. This voltage was maintained for 43h and then the column was emptied at 80ml/h. The supernatant aspartate transaminase and malate dehydrogenase were totally separated by this procedure. A typical procedure is shown in Table 2.

Electrophoresis

On starch gels, dolphin muscle alanine transaminase appeared as a single band migrating 2.5cm towards the anode in both crude and purified preparations.

The purification of aspartate transaminase was monitored by starch-gel electrophoresis (described in the Materials and Methods section) and only two forms of the enzyme were observed. The cationic form migrated 3.0cm towards the cathode whereas the anionic form migrated 2.5cm towards the anode in both crude and purified preparations. There appeared to be an equal distribution of these two forms.

Starch-gel electrophoresis of crude dolphin muscle preparations by the methods of Bertland & Kaplan (1968) and polyacrylamide disc-gel electrophoresis of the purified preparations by the method of Davis (1964) further demonstrated that both the supernatant and the mitochondrial enzymes occurred as single forms. The disc gels also revealed that the final supernatant and mitochondrial enzyme preparations were not completely homogeneous, containing one and two contaminating proteins respectively.

Molecular-weight determinations

For comparative purposes, the apparent molecular weights of aspartate transaminase and malate dehydrogenase from a variety of sources are listed in Table 3.

Effect of pH

The optimum activity of alanine transaminase occurred between pH7.15 and 7.8. At pH6.3, 6.8 and 7.3 the relative activities were 40, 75 and 100% respectively.

The optimum activity of the supernatant mitochondrial aspartate transaminases occurred at pH7.3–8.3 and pH6.3–7.3 respectively. At pH7.3 both forms were capable of 95% of their maximum activity. At pH6.8 this value was 76 and 100% for the supernatant and mitochondrial enzymes respectively.

Enzyme kinetics

For alanine transaminase, a decrease in the assay pH from 7.3 to 6.3 caused an increase in the apparent K_m values of both amino acids, a decrease in the apparent K_m value of pyruvate, and no change in the apparent K_m value of α -oxoglutarate (Table 4). Absolute K_m values for each substrate at infinite concentrations of co-substrate were obtained by plotting $1/K_m$ versus $1/[\text{co-substrate}]$ (Hopper & Segal, 1962) and by extrapolating to $1/[\text{co-substrate}]$ equals zero (Table 4). When $1/K_m$ values for α -oxoglutarate, glutamate and pyruvate were plotted against $1/[\text{alanine}]$, $1/[\text{pyruvate}]$ and $1/[\text{glutamate}]$ respectively, the expected straight lines with positive slopes were obtained which show that the K_m value increases with increasing concentrations of co-substrate. However, when the $1/K_m$ values of alanine were plotted against $1/[\alpha\text{-oxoglutarate}]$ a U-shaped curve resulted, illustrating that, above 2.5 mM- α -oxoglutarate, increased α -oxoglutarate concentrations caused a sharp decrease in the K_m value of alanine. The cause of this effect is not known.

The aspartate transaminase kinetics showed that the supernatant enzyme had a much higher affinity for α -oxoglutarate and glutamate, a slightly higher affinity for oxalacetate and a much lower affinity for aspartate than the mitochondrial form at both pH 6.8 and 7.3 (Table 5). When the assay pH was decreased from 7.3 to 6.8, the apparent K_m values of both amino acids increased whereas the apparent K_m values of both α -oxo acids decreased for both aspartate transaminases as observed with alanine transaminase (Table 4). The apparent equilibrium constant (K_{eq}) was not affected by pH but mitochondrial aspartate transaminase favoured the production of α -oxo-glutarate and aspartate more than the supernatant form (Table 6).

Inhibition of aspartate transaminase

The two isoenzymes were assayed in both directions with the following metabolites: AMP, 0.5 mM; cyclic AMP, 0.2 mM; ADP, 1.0 mM; ATP, 1.0 mM; aspartate, 24 mM; creatine phosphate, 2.5 mM; fumarate, 1.0 mM; glutamate, 5.0 mM; glyoxylate, 1.0 mM; α -oxoglutarate, 3 mM; DL-lactate, 20 mM; L-malate, 3 mM; pyruvate, 1.0 mM; NAD, 0.5 mM; succinate, 3 mM. Only succinate and glutamate were

Table 3. Molecular weights obtained by Sephadex G-200 chromatography

A column (1.6 cm $\times$ 33 cm) of Sephadex G-200 was equilibrated with buffer C plus 0.02% sodium azide and calibrated with Dextran Blue 2000 (1 mg/ml), aldolase plus chymotrypsin A (5 mg of each/ml) and ovalbumin plus ribonuclease A (5 mg of each/ml), all from the calibration kit supplied by Pharmacia Fine Chemicals. The molecular weights of the unknown enzymes were calculated by the method of Granath & Kvist (1967). All samples (0.4 ml) were subjected to ascending chromatography at a flow rate of 6 ml/h. The reported values are the means of duplicate determinations and the differences between duplicates did not exceed 1.5%. All enzyme assays were by the standard method at pH 7.3.

Sample	Apparent molecular weight
Purified dolphin muscle supernatant aspartate transaminase	79 000
Malate dehydrogenase in supernatant transaminase preparation	80 100
Sigma pig heart aspartate transaminase	76 200
Sigma pig heart malate dehydrogenase	57 400
Aspartate transaminase in crude dolphin muscle preparation	75 900
Malate dehydrogenase in crude dolphin muscle preparation	64 600

Table 4. K_m values for purified dolphin muscle alanine transaminase

The K_m values of each substrate at infinite concentration of its co-substrate was calculated as described in the text of the Results section. For α -oxoglutarate, K_m values were determined at 5, 10, 25 and 100 mM-alanine by using 0.1–5 mM- α -oxoglutarate; for alanine, at 0.5, 1.0, 1.5, 2.5, 5.0 and 10 mM- α -oxoglutarate by using 3–100 mM-alanine; for pyruvate, at 2.5, 7.5 and 25 mM-glutamate by using 0.05–2.0 mM-pyruvate; for glutamate, at 0.5, 1.5 and 5.0 mM-pyruvate by using 1.25–25 mM-glutamate. All K_m values at pH 6.3 were determined at the concentration of co-substrate that was saturating or very nearly saturating at pH 7.3. All assays were by the standard method.

Substrate Co-substrate	K_m values of substrate (mM)							
	α -Oxoglutarate		Alanine		Pyruvate		Glutamate	
	Alanine		α -Oxoglutarate		Glutamate		Pyruvate	
Concentration of co-substrate (mM)	25	∞	1.0	∞	25	∞	5.0	∞
pH 7.3	0.45	0.56	8.2	9.4*	0.87	1.1	15	17
pH 6.3	0.44		34		0.49		44	

* This value is in some doubt as a plot of $1/K_m$ (alanine) versus $1/[\alpha\text{-oxoglutarate}]$ was not linear, as described in the text.

Table 5. *K_m* values for dolphin muscle aspartate transaminase

All *K_m* values were determined in duplicate at the concentration of co-substrate that was saturating or very nearly saturating at pH7.3 by using the standard method.

		<i>K_m</i> values of substrate (mM)							
Substrate	...	α-Oxoglutarate		Aspartate		Oxaloacetate		Glutamate	
Co-substrate	...	Aspartate		α-Oxoglutarate		Glutamate		Oxaloacetate	
		Mito- chondrial	Super- natant	Mito- chondrial	Super- natant	Mito- chondrial	Super- natant	Mito- chondrial	Super- natant
Concentration of co- substrate (mM)		10	10	5	2	24	12	0.4	0.4
Mitochondrial	pH7.3	0.76		0.50		0.10		9.4	
	pH6.8	0.68		0.65		0.06		11.0	
Supernatant	pH7.3		0.13		2.4		0.06		3.2
	pH6.8		0.09		3.7		0.05		4.6

Table 6. Apparent equilibrium constants (*K'_{equil.}*) for purified dolphin muscle aspartate transaminase

Purified aspartate transaminase (2units in 0.1ml) was added to 4.9ml of 100mM-sodium phosphate buffer containing 7.5μmol each of α-oxoglutarate and aspartate. Portions (0.75ml) were added to 7.5ml of 10% (w/v) trichloroacetic acid after 15 and 20min. The precipitates were sedimented and portions of the supernatants were transferred to cuvettes, neutralized with 1M-NaOH and assayed for α-oxoglutarate, oxaloacetate and aspartate content by the standard method at pH7.3. The α-oxoglutarate assay contained 0.2ml of trichloroacetic acid supernatant, 16mM-(NH₄)₂SO₄ and 6 units of glutamate dehydrogenase; the oxaloacetate assay, 0.5ml of trichloroacetic acid supernatant and 10units of malate dehydrogenase; the aspartate assay, 0.2ml of trichloroacetic acid supernatant, 50mM-α-oxoglutarate, 10units of malate dehydrogenase and 5units of Sigma aspartate transaminase. The *E*₃₄₀ was determined before the addition of enzyme, after the reaction had gone to completion and after the addition of another portion of enzyme to allow correction for the absorbance change caused by the addition of enzyme. Glutamate production was assumed to equal oxaloacetate production. Each *K'_{equil.}* was determined in duplicate and calculated from the equation:

$$K'_{equil.} = \frac{[\alpha\text{-oxoglutarate}][\text{aspartate}]}{[\text{oxaloacetate}][\text{glutamate}]}$$

by using the 20min values which represented equilibrium in all cases.

Enzyme	pH	<i>K'_{equil.}</i>
Mitochondrial	7.3	9.27
	6.8	9.18
Supernatant	7.3	6.77
	6.8	6.90

inhibitory in the direction of glutamate production whereas only malate and α-oxoglutarate were inhibitory in the direction of α-oxoglutarate production (Table 7). Inhibition was more effective at pH6.8 than at pH7.3 in all cases except for the

inhibition of the mitochondrial form by glutamate and α-oxoglutarate. The supernatant form was more sensitive to inhibition by succinate, glutamate and α-oxoglutarate whereas the reverse was true with malate.

Substrate inhibition by oxaloacetate and α-oxoglutarate was also examined (Table 8). Again, inhibition was greater at pH6.8 than at pH7.3 and the supernatant form was inhibited to a greater extent by both substrates.

Aspartate transaminase specificity

No measurable transamination of the α-oxo acids of tyrosine, leucine or phenylalanine with glutamate was observed with either isoenzyme.

Discussion

Comparative aspects of the transaminases

The purification procedures for the transaminases did not provide completely homogeneous preparations since they were primarily chosen to eliminate, in a minimum number of steps, contaminating enzymes that utilize the same substrates as the transaminases. However, the purification of alanine transaminase (Table 1) compared well with the degree of purification obtained in other laboratories although the specific activities of our preparations were lower (Gatehouse *et al.*, 1967; Matsuzawa & Segal, 1968; Saier & Jenkins, 1967; Segal *et al.*, 1962). Our aspartate transaminase (Table 2) also was comparable in specific activity and degree of purification achieved in other laboratories (Aoki *et al.*, 1972; Bertland & Kaplan, 1968; Krista & Fonda, 1973; Magee & Phillips, 1971; Martinez-Carrion *et al.*, 1965; Shrawder & Martinez-Carrion, 1973). The absence of subforms of the three purified transaminases contrasts with the results of other workers who have demonstrated multiple forms of these enzymes

Table 7. *Inhibition of purified dolphin muscle aspartate transaminase by dicarboxylates*

The substrate concentrations used approximate to the K_m values listed in Table 5 except for oxaloacetate since assays at K_m concentrations of this substrate were non-linear with time. All assays were by the standard method except for those used to study α -oxoglutarate inhibition. These assays contained, in a final volume of 2.5 ml, the indicated concentrations of substrate but no NADH or coupling enzyme. The rate of enzyme activity was determined by following the disappearance of oxaloacetate at 280 nm. The concentrations of inhibitors were varied from 0 to 25 mM and the concentration required for 50% inhibition was determined by Dixon plots of $1/v$ versus $[I]$.

Enzyme	pH of assay	Substrate concentration (mM)		Inhibitor concentration required for 50% inhibition (mM)	
		α -Oxoglutarate	Aspartate	Succinate	Glutamate
Mitochondrial	7.3	0.73	0.50	70	24
	6.8	0.73	0.50	31	24
Supernatant	7.3	0.15	3.0	40	10
	6.8	0.08	3.0	14	7.2
		Oxaloacetate	Glutamate	Malate	α -Oxoglutarate
Mitochondrial	7.3	0.10	10.0	6.0	15
	6.8	0.10	10.0	2.9	19
	7.3	0.10	4.0	9.0	3.2
Supernatant	6.8	0.10	4.0	5.0	2.2

Table 8. *Inhibition of purified dolphin muscle aspartate transaminase by α -oxoglutarate and oxaloacetate*

All assays were conducted by the standard method at the indicated amino acid concentrations and α -oxo acid concentrations of 0.1–15 mM. The amino acid concentrations approximate the K_m values listed in Table 5.

Enzyme	pH of assay	Concentration of amino acid substrate (mM)	Inhibition at 5 mM concentration of α -oxo acid substrate (%)	
			Oxaloacetate	α -Oxoglutarate
Mitochondrial	7.3	Glutamate (10)	17.5	
	6.8		36.6	
	7.3	Aspartate (0.6)		0.00
	6.8			5.9
Supernatant	7.3	Glutamate (4)	21.5	
	6.8		41.2	
	7.3	Aspartate (3)		18.5
	6.8			38.6

(Bertland & Kaplan, 1968, 1970; Chen *et al.*, 1973; Gatehouse *et al.*, 1967; Krista & Fonda, 1973; Magee & Phillips, 1971; Martinez-Carrion *et al.*, 1967; Martinez-Carrion & Tiemeier, 1967; Michuda & Martinez-Carrion, 1969; Shrawder & Martinez-Carrion, 1973) but is in agreement with those who have observed only single forms (Bertland & Kaplan, 1970; Magee & Phillips, 1971; Saier & Jenkins, 1967). The alanine transaminase is assumed to be cytoplasmic since the mitochondrial form of this enzyme is either extremely labile (Sallach & Fahien, 1969) or non-existent (Davis, 1968).

The very similar molecular weights of malate dehydrogenase and supernatant aspartate transaminase (Table 3) explains the co-purification of these two enzymes, a situation that has been observed in other laboratories (Krista & Fonda, 1973; Martinez-Carrion & Tiemeier, 1967). However, the values of 80000 are unusual for both enzymes since the

molecular weight of malate dehydrogenase is about 60000 (Murphy *et al.*, 1967) and that of aspartate transaminase is 90000–110000 (Krista & Fonda, 1973) although Magee & Phillips (1971) obtained a value of 79000 for rat brain transaminase. The molecular weight estimates of 60000 obtained for malate dehydrogenase in the crude dolphin muscle preparation and the mixture of Sigma pig heart enzymes illustrate that the high-molecular-weight malate dehydrogenase in the supernatant transaminase preparation either arose as an artifact during the purification or represents a different species of this enzyme. The consistently low apparent molecular weight of the transaminase from all sources probably results from the characteristic of molecular sieves to measure Stokes radii rather than true molecular weights. Since the dolphin muscle aspartate transaminase in both the purified and crude dolphin muscle preparations migrated through the gel at the

same rate as the Sigma pig heart transaminase it is concluded that the true molecular weight of both dolphin muscle enzymes is probably identical with the value of 110000 found for the pig heart enzyme (Michuda & Martinez-Carrion, 1969; Jenkins *et al.*, 1959). It is not clear why the values found for aspartate transaminase in the present study differ from those determined by other workers using Sephadex G-200 (Krista & Fonda, 1973; Michuda & Martinez-Carrion, 1969).

Roles of the transaminases in the diving mammal

The role of the aspartate and alanine transaminases in the anaplerotic maintenance of tricarboxylic acid-cycle intermediates is well established (Davis *et al.*, 1972; Davis & Bremer, 1973; Lowenstein, 1972; Safer *et al.*, 1971; Safer & Williamson, 1973) and we believe this function to be of particular importance to diving mammals since the transition from anaerobic to aerobic metabolism in these animals involves a shift from carbohydrate fermentation to fat oxidation. Such a shift causes a momentary piling up of acetyl-CoA until oxaloacetate reserves are augmented by the transamination of aspartate through the combined action of these two enzymes (Safer & Williamson, 1973). The dolphin muscle transaminases seem particularly well suited to this function since their activities are up to 15-fold higher than those found in other mammalian muscles (Zimmerman *et al.*, 1968). Also, the mitochondrial aspartate transaminase has a high affinity for aspartate (Table 5), a K_m for oxaloacetate that is 10-fold higher than physiological concentrations and, in the reverse direction, is inhibited by malate (Table 7) which increases in concentration during tricarboxylic acid-cycle activation (Safer & Williamson, 1973). This inhibition is potentiated by the low pH values which would occur at the end of a dive.

Another well-established role of aspartate transaminase is its function in the malate-aspartate cycle (Krebs & Veech, 1969; LaNoue *et al.*, 1973; Safer *et al.*, 1971; Safer & Williamson, 1973). Since diving animals endure severe drops in NAD^+/NADH ratios (Lai & Miller, 1973) this cycle should be very active. This supposition is supported by the observation that dolphin muscle has (1) high aspartate transaminase activity as mentioned above, (2) approximately equal distribution of the two aspartate transaminases according to the separations by electrophoresis and CM-cellulose (Table 2) and (3) a mitochondrial isoenzyme that favours aspartate production more than the supernatant form (Table 5).

In addition to these two familiar functions of the transaminases, we believe that these enzymes catalyse the first steps in a pathway that could produce ATP from the carbon skeletons of amino acids during hypoxia (Scheme 1). The model is based on the

substrate-level phosphorylation of GDP at the succinyl thiokinase step of the tricarboxylic acid cycle being coupled by NAD^+ and NADH to the energy-producing reduction of fumarate to succinate by a reversal of the succinate dehydrogenase reaction. This latter reaction scheme has been observed in mammalian tissues (Chance & Hollunger, 1960; Penney & Cascarano, 1970; Randall & Cohen, 1966; Sanadi & Fluharty, 1963; Wilson & Cascarano, 1970). When these two reactions are coupled as shown in Scheme 1, redox balance within the mitochondria is maintained and succinate accumulates as has been observed during hypoxia in mammalian tissues (Hoberman & Prosky, 1967; Kondrashova & Chahovets, 1971; Mensen de Silva & Cazorla, 1973; Weil-Malherbe, 1937). The succinate can pass out of the mitochondria in an exchange for malate involving phosphate and in direct exchange for α -oxoglutarate (Chappell, 1968; Klingenberg, 1970*a, b*) and may accumulate in sufficient quantities in diving mammals (Hochachka *et al.*, 1974) to exert some control over the supernatant aspartate transaminase reaction as a product inhibitor (Table 7). Fumarate for the reversed succinate dehydrogenase reaction could be supplied by the transamination of aspartate in the cytosol to form oxaloacetate which is then reduced to malate by malate dehydrogenase. The malate passes into the mitochondria in exchange for succinate as mentioned above and is converted into fumarate by fumarase, which is freely reversible. The α -oxoglutarate for the succinyl thiokinase reaction could be supplied by the transamination of glutamate by alanine transaminase, and α -oxoglutarate for the supernatant aspartate transaminase reaction could come from endogenous pools until a cycling of this metabolite and glutamate between these two enzymes could be established as described by Safer & Williamson (1973). The required aspartate and glutamate could be derived from the pools of free amino acids. Indeed, aspartate concentrations have been observed to decrease under conditions which enhance glycolysis in the tissues of mammals (Brosnan *et al.*, 1970; Gailis & Benmouyal, 1973; Hochachka *et al.*, 1974). On the basis of enzyme activities, pyruvate is considered to be the primary acceptor of glutamate nitrogen since, other than pyruvate and oxaloacetate, only the α -oxo acid of tyrosine participates in the transaminase reaction with glutamate in crude dolphin muscle preparations. This activity is very low despite the fact that the particulate fraction of the homogenate, which contains significant transaminase activities (Cammarata & Cohen, 1950; Hird & Roswell, 1950), was included in the assays. The small amount of tyrosine transaminase activity observed was probably not due to a non-specific reaction of aspartate transaminase since, unlike this enzyme from other

Scheme 1. A metabolic map indicating the postulated role of aspartate and alanine transaminases during anaerobic excursions in the diving mammal

Aspartate mobilization is thought to lead to an energy-yielding reduction of fumarate, whereas α -oxoglutarate derived from alanine transaminase reaction is presumably converted into succinyl-CoA, a process setting the stage for a substrate-level phosphorylation. The two pathways are thought to be linked functionally by the formation of a redox couple between fumarate reduction and the α -oxoglutarate dehydrogenase reaction and by the cycling of α -oxoglutarate and glutamate between the two transaminases.

sources (Miller & Litwack, 1971; Shrawder & Martinez-Carrion, 1972), both dolphin muscle isoenzymes were totally inactive with the α -oxo acids of tyrosine, phenylalanine and leucine. Alanine

transaminase activity in dolphin muscle, however, is quite high (16 units/g of tissue) compared with that found in the muscle of other mammals (Zimmerman *et al.*, 1968). This activity is definitely not due to

Table 9. *Apparent equilibrium constants ($K'_{\text{equil.}}$) of dolphin and rabbit muscle lactate dehydrogenases*

Dolphin muscle was homogenized in 100mM-sodium phosphate, pH 6.8, (4 ml/g of tissue) and centrifuged at 30000g for 30 min. Rabbit muscle lactate dehydrogenase from Sigma was diluted 10-fold to yield 750 units/ml. Portions (0.02 ml) of these preparations were added to cuvettes containing 2.25 ml of 30 mM-L-lactate + 5 mM-NAD⁺ in 100 mM-sodium phosphate buffer and the reaction was allowed to go to completion. The E_{340} was recorded before the addition of enzyme, after the reaction had gone to completion and after the addition of another portion of enzyme to allow correction for the absorbance change caused by the addition of enzyme. $K'_{\text{equil.}}$ was determined by using the equation:

$$K'_{\text{equil.}} = \frac{[\text{pyruvate}][\text{NADH}]}{[\text{lactate}][\text{NAD}^+]}$$

Source of lactate dehydrogenase	pH	...	6.3	$K'_{\text{equil.}} \times 10^4$ 6.8	7.0	7.3
Dolphin muscle			0.21	0.62	1.08	1.96
Rabbit muscle			0.18	0.58	0.98	1.75

aspartate transaminase either, since the two activities can be totally separated by (NH₄)₂SO₄ fractionation (Tables 1 and 2). Further support for the contention that pyruvate acts as the primary acceptor for glutamate nitrogen comes from the observation that alanine accumulates as an end-product when glycolysis is stimulated in mammalian tissues (Brosnan *et al.*, 1970; Felig & Wahren, 1971; Gailis & Benmouyal, 1973; Hochachka *et al.*, 1974).

If pyruvate is to play the key role indicated in Scheme 1, a continuous source must be ensured. It is suggested that glucose is the source of pyruvate since there was little or no measurable 'malic' enzyme activity in the dolphin muscle. Although Reeves (1963) reported that essentially 100% of labelled glucose was converted into lactate in turtle hearts during hypoxia, Brachfeld *et al.* (1972) reported a lower value for the same diving species. In view of their results it seems unlikely that such total conversion of glucose into lactate represents the only possibility. Also, the data of Reeves (1963) would indicate that the only pathway for the regeneration of NAD⁺ consumed by glycolysis would be the lactate dehydrogenase reaction. In Scheme 1 it is apparent that the malate dehydrogenase reaction could provide NAD⁺ for glycolysis as could the succinate-stimulated fatty acid synthesis observed during hypoxia (Brachfeld *et al.*, 1972; Cherchi *et al.*, 1970; Whereat *et al.*, 1967). These additional reducing equivalents could presumably free some pyruvate for transamination with glutamate. Indeed, it has been reported that under conditions of glycolytic activation in mammalian tissues, (1) a large fraction of the NAD⁺ needed for glycolysis is supplied by malate dehydrogenase (Safer & Williamson, 1973), (2) amino acids are in equilibrium with their α -oxo acids, (3) the specific radioactivity of alanine approaches that of lactate (Gailis & Benmouyal, 1973), and (4) alanine accounts for 12–18% of the glucose consumed (Felig & Wahren, 1971). As these conditions continue, the NAD⁺/NADH ratio and the pH value both decrease (Chance

et al., 1964), which drive, the lactate dehydrogenase in dolphin muscle farther towards the production of lactate (Table 9). This effect must be countered by mass-action effects of lactate accumulation since pyruvate concentrations actually increase during hypoxia (Edington *et al.*, 1973; Hochachka *et al.*, 1974) and may approach one-twentieth the concentration of lactate (White & Kaplan, 1972). Also, since the K_m value of pyruvate for alanine transaminase decreases with pH (Table 4), this enzyme effectively competes for pyruvate throughout the duration of hypoxia. This conclusion is confirmed by the above-mentioned alanine accumulation observed in vertebrate tissues. Another effect of the decrease in pH is that the K_m of α -oxoglutarate for the supernatant aspartate transaminase is decreased (Table 5) whereas succinate inhibition of this enzyme is enhanced to a point where it is as effective as glutamate as an inhibitor (Table 7). Since glutamate concentrations would presumably fall as α -oxoglutarate was produced by alanine transaminase [observed by Gailis & Benmouyal (1973), but not by Brosnan *et al.*, (1970)], it seems possible that succinate and glutamate exert a reciprocal control over the supernatant aspartate transaminase reaction. The significance of the remainder of the inhibitions by metabolites listed in Tables 7 and 8 is difficult to ascertain at this time but it seems likely that pools of these intermediates could be instrumental in the controlling of the direction of the aspartate transaminase reaction as suggested by Michuda & Martinez-Carrion (1970).

In conclusion, we suggest that the alanine and aspartate transaminases are particularly important in the intermediary metabolism of diving mammals since they perform key functions during most of the metabolic conditions that the animals encounter. These functions are (1) anaplerotic maintenance of tricarboxylic acid-cycle intermediates under all conditions but particularly during intense lipid mobilization at the end of a dive, (2) restoration of redox balance after hypoxia and (3) the enhancement of ATP production during hypoxia.

This work was supported by operating grant no. 67-3678 to P. W. H. from the National Research Council of Canada and was taken from a Ph.D thesis by T. G. O. who is a recipient of a fellowship from the Fisheries Research Board of Canada, block grant no 65-1848.

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