Differences in 2-oxoglutarate dehydrogenase regulation in liver and kidney

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In response to acidosis, renal ammoniogenesis is stimulated, enhancing urinary buffering power, while hepatic ammoniogenesis and ureagenesis decrease, so as to spare bicarbonate consumed in the urea cycle. 2-Oxoglutarate (2-OG) levels can regulate ammoniogenesis in kidney and gluconeogenesis in liver and kidney. Since the activity of 2-oxoglutarate dehydrogenase (2-OGDH) has an important influence on cellular levels of 2-OG, this study evaluated the effects of pH on 2-OGDH in liver and kidney and found that: (1) the isolated enzyme from both organs has the same pH-sensitivity; (2) 2-OGDH flux measured in intact mitochondria was inhibited by increasing H^+ in liver, but stimulated in kidney; (3) transport of 2-OG into the mitochondria was not rate-limiting; (4) liver mitochondrial 2-OGDH exhibited a strong preference for 2-OG generated within the mitochondria from glutamate–oxaloacetate transaminase (GOT), suggesting that channelling between GOT and 2-OGDH occurs. Since complexation between 2-OGDH and GOT occurs *in vitro*, we propose that the degree of complexation is higher in liver than in kidney, such that most of the 2-OGDH may be complexed to GOT in liver. In the liver the inherent H^+ -sensitivity of 2-OGDH is masked by the pH-sensitivity of GOT and the glutamate–aspartate carrier.

INTRODUCTION

Renal tubular cells and liver parenchymal cells share many of the same enzymes and metabolic pathways. Although similar enzymes and reaction sequences are involved in the control of acid–base balance in the two organs, the response of the kidney and liver to acidosis differs markedly. The kidney responds to acidosis by increasing ammonia production and gluconeogenesis from glutamine. This results in increased secretion of acid by the kidney, but, in contrast, the liver responds to acidosis by decreasing ammonia production for urea synthesis (Haussinger *et al.*, 1984) and decreasing gluconeogenesis. This conserves the bicarbonate normally consumed in the urea cycle. Residual ammonia is converted into glutamine, which provides additional substrate to the kidney for ammoniogenesis. Thus the two organs work in concert to restore acid–base homeostasis during periods of acidosis.

The level of 2-OG in the cell inhibits the ammoniagenic and gluconeogenic mitochondrial enzymes phosphate-dependent glutaminase and glutamate dehydrogenase (Preuss *et al.*, 1974), and current evidence suggests that it plays an important regulatory role in the formation of ammonia in the kidney (Tannen & Kunin, 1976) and in gluconeogenesis in the liver (Sterniczuk *et al.*, 1991). The tricarboxylic-acid-cycle enzyme 2-oxoglutarate dehydrogenase (2-OGDH) is responsible for the oxidation of 2-OG, and its activity has a strong influence on cellular levels of 2-OG. The K_m of renal 2-OGDH falls in response to a fall in pH (Lowry & Ross, 1980), inducing a decrease in 2-OG levels in the acutely acidotic kidney (Tannen & Kunin, 1976). Schoolwerth & LaNoue (1984) demonstrated with isolated renal mitochondria that the H^+ -induced decrease in 2-OG was the key factor in causing the H^+ -induced increase in ammoniogenesis from glutamine. The impact of acidosis on liver 2-OGDH has not been fully clarified. However, isolated liver mitochondrial ammonia production from glutamate is known to be as sensitive to 2-OG levels as is kidney mitochondrial ammonia production (LaNoue

et al., 1983). Recent studies show that after treatment with Ca^{2+} -mobilizing hormones a 5-fold decrease in liver 2-oxoglutarate levels occurs owing to Ca^{2+} stimulation of 2-OGDH (Strzelecki *et al.*, 1988; Sterniczuk *et al.*, 1991). This decrease in 2-OG appears to be responsible for the stimulation of gluconeogenesis, just as the stimulation of gluconeogenesis in the acidotic kidney can be attributed to the fall in 2-OG levels.

Since studies of the isolated 2-OGDH enzyme in a variety of organs show that both Ca^{2+} and H^+ dramatically lower the K_m of the enzyme for 2-oxoglutarate (McCormack & Denton, 1979; Lowry & Ross, 1980; Rutter & Denton, 1988), it is strange that increases in intracellular free Ca^{2+} stimulate gluconeogenesis and ureagenesis, but increases in H^+ do not. Clearly, in the kidney, 2-OG levels, ammonia production and gluconeogenesis respond in the same manner to $[H^+]$ and to increases in intracellular Ca^{2+} brought about by Ca^{2+} -mobilizing hormones, and to increases in H^+ (Kamm *et al.*, 1967; Kurokawa & Massry, 1973; Lowry & Ross, 1980; Schoolwerth & LaNoue, 1983). There are three possible explanations for this interesting dichotomy. The first possibility is that the enzymes in the two organs differ in their sensitivities to pH and Ca^{2+} . The second possibility is that the rate of transport of 2-OG into mitochondria is rate-limiting and differs in the two organs. The third possibility is that an enzyme complex between glutamate–oxaloacetate transaminase (GOT) and 2-OGDH, which has been reported to occur in liver mitochondria *in vitro* (Fahien *et al.*, 1988), is responsible for the differing sensitivities. The present study was undertaken to evaluate systematically which of these possibilities accounts for the differences in ammoniagenic response between the two organs.

METHODS

Preparation of mitochondria

The liver or kidney from male Sprague–Dawley rats was perfused *in situ* with a cold medium (MSE) containing 225 mM-

Abbreviations used: 2-OG, 2-oxoglutarate; 2-OGDH, 2-OG dehydrogenase; GOT, glutamate–oxaloacetate transaminase.

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mannitol, 75 mM-sucrose, 4 mM-EGTA and 5 mM-Mops. The organ was then removed from the animal, and liver or kidney cortical mitochondria were prepared by standard techniques of differential centrifugation (Schneider & Hogeboom, 1950; Schoolwerth *et al.*, 1978). Respiratory control ratios were determined as previously described at 28 °C (Schoolwerth *et al.*, 1978). Mitochondria with RCRs less than 5 were discarded.

Measurement of intramitochondrial Ca^{2+}

Intramitochondrial free Ca^{2+} concentration was determined by using the fluorescent Ca^{2+} indicator fura-2 {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy)ethane-*NNN'*-tetra-acetic acid; excitation at 340 and 380 nm, emission at 510 nm}. Mitochondria were loaded with fura-2 as previously described (Wan *et al.*, 1989). RCRs of the loaded mitochondria (6.41 ± 0.11) were not different from control RCRs (6.25 ± 0.28).

The apparent dissociation constant (K_d) of intramitochondrial fura-2 was determined in incubation medium (145 mM-KCl, 20 mM-Mops, 10 mM-Tris, 5 mM- KH_2PO_4 , 2 mM-EGTA and 1 mM- MgCl_2 ; medium A) as previously described (Wan *et al.*, 1989). The K_d of fura-2 in liver and kidney mitochondria was measured to be 0.155 μM , in agreement with other published results (Gunter *et al.*, 1988). This value of K_d was used to calculate matrix $[\text{Ca}^{2+}]$ in fura-2-loaded mitochondria, according to the equation of Grynkiewicz *et al.* (1985). No correction was made for autofluorescence, as it contributed < 1% of total measured fluorescence intensity of fura-2-loaded mitochondria.

Determination of intramitochondrial pH

Mitochondria were loaded with the acetoxymethyl ester (10 μM) of the pH-sensitive indicator BCECF (carboxyethyl-5,6-carboxyfluorescein; excitation at 500 nm, emission at 530 nm), washed, and resuspended in medium B (see below) at pH 6.6 or 7.0. After correction for dye leakage and autofluorescence, matrix pH was determined from a calibration curve generated by incubating dye-loaded mitochondria in medium B at various pH values between 6.4 and 7.8 in the presence of valinomycin (0.5 $\mu\text{g}/\text{ml}$) and the uncoupling agent 1799 (1 nmol/mg of mitochondrial protein).

In the liver, the intramitochondrial pH values measured in medium B were 7.07 ± 0.07 and 7.64 ± 0.05 when the outside pH values were 6.6 and 7.0 respectively. In the kidney, the intramitochondrial pH values were 7.28 ± 0.01 and 7.64 ± 0.02 when the outside pH values were 6.6 and 7.0 respectively. Addition of Ca^{2+} , substrates and/or ADP resulted in changes in intramitochondrial pH of less than 0.04 pH unit.

Measurement of 2-OGDH activity in permeabilized mitochondria

The kinetic parameters of 2-OGDH in permeabilized mitochondria were measured as described by Denton and co-workers (McCormack & Denton, 1979; Thomas & Denton, 1986; Rutter & Denton, 1988) with some modifications. Kidney and liver mitochondria were incubated with Triton X-100 (0.09%, v/v), rotenone (1.5 $\mu\text{g}/\text{mg}$ of mitochondrial protein), 1 mM- NAD^+ , 2-OG (0–5 mM), 0.3 mM-CoA and 3 mM-DL-dithiothreitol in a buffer (pH 7.1 or 7.7) consisting of 140 mM-KCl, 20 mM-Mops, 10 mM-Tris, 1 mM-EGTA, 10 mM- KH_2PO_4 and 3 mM-malate. 2-OGDH activity (28 °C) was monitored fluorometrically as initial rates of NADH production (excitation at 340 nm, emission at 478 nm). Assays were initiated by addition of CoA. Rates of NADH oxidation in the absence of CoA were subtracted from those measured in its presence to obtain enzyme activity. Enzyme

activity was similar when Triton X-100 was varied between 0.06 and 0.15%.

Measurement of 2-OG oxidation

Intact mitochondria isolated from liver and kidney were first depleted of endogenous substrates by including 2 mM-ADP in the isolation media during the final centrifugal rinse. Mitochondria were resuspended in isolation media with 2 mM-ADP and then incubated in medium A also containing 2 mM-ADP, 30 mM-malate and various concentrations of 2-OG at pH 6.6 and 7.0. Oxygen consumption was measured polarographically with a Clark-type electrode. At 28 °C, in the absence of added 2-OG, the rate of endogenous respiration by liver mitochondria was less than 3 ng-atoms of O/min per mg of mitochondrial protein. Since malate blocks oxidation of succinate, there was a 1:1 stoichiometry between nmol of 2-OG oxidized and ng-atoms of oxygen consumed.

Measurement of 2-OGDH flux

Mitochondria were incubated at 28 °C in a buffer (medium B) containing 145 mM-KCl, 10 mM-Tris, 20 mM-Mops, 10 mM- KH_2PO_4 , 1 mM-EGTA, 2.5 mM- MgCl_2 , 1 mM-DL-cycloserine, 5 mM-malate, 1 mM-[2,3- ^3H]glutamate and 0.5 mM-2-[U- ^{14}C]oxoglutarate. Buffer pH, free Ca^{2+} concentration and ADP concentrations were varied according to experimental protocol. HClO_4 (14%) -extracted samples taken at 0, 2, 4, 6 and 8 min were passed through Dowex-1 formate columns (Bio-Rad AG 1-X10) to separate mitochondrial metabolites as described previously (LaNoue *et al.*, 1970). The single peak containing aspartate and glutamate was placed on a Dowex-1 acetate column (Berl *et al.*, 1961) to separate the two amino acids. Radioactivity was determined in each peak by double-label counting. The d.p.m. corrected for metabolic losses of $^3\text{H}_2\text{O}$ or $^{14}\text{CO}_2$ were converted into nmol of product by dividing the corrected d.p.m. values by the initial specific radioactivity of either [^3H]glutamate or 2-[^{14}C]OG. Corrections for loss of $^3\text{H}_2\text{O}$ or $^{14}\text{CO}_2$ were accomplished by one of the following.

1. Multiplying the d.p.m. in [^3H]2-OG by 1.5. This corrects for the loss of one of the three ^3H atoms in [2,3- ^3H] glutamate when that metabolite is converted into 2-oxo[3- ^3H]glutarate.
 2. Multiplying the d.p.m. in [^3H]succinate by 1.5. No ^3H is lost when 2-oxo[3- ^3H]glutarate is converted into succinate.
 3. Multiplying the d.p.m. in [^3H]malate by 3. Only one of the original three ^3H atoms in [2,3- ^3H]glutamate is retained in the conversion of glutamate into malate.
 4. Multiplying the d.p.m. in [^{14}C]succinate and malate by 1.25 to compensate for the loss of one of the five ^{14}C atoms in [U- ^{14}C]2-OG during the decarboxylation of 2-OG to succinate.
- Aspartate and 2-oxoglutarate were assayed by standard nicotinamide-nucleotide-linked enzymic reactions (Williamson & Corkey, 1969; Lowry & Passoneau, 1972). Protein was measured by a biuret assay as previously described (Cleland & Slater, 1953).

Total 2-OGDH flux was calculated from changes (Δ) in 2-OG and aspartate (ASP) levels over an 8 min incubation, according to the equation:

$$\text{Flux} = \Delta \text{ASP} - \Delta 2\text{-OG}.$$

In preliminary experiments with 1 mM-malate as substrate, about 2.5% of the radiolabelled products of 2-OGDH accumulated as aspartate. When studies were conducted with 5 mM-malate as substrate, no measurable radiolabelled aspartate was detected, since the radiolabelled products of 2-OGDH were trapped in malate and succinate. To determine the rate of [^3H]2-OG oxidation, the amounts of [^3H]succinate and [^3H]malate at 0, 2 and 4 min were determined. Oxidation of [^{14}C]2-OG was calculated similarly.

Materials

Radiochemicals ($[^{14}\text{C}]$ 2-OG, $[^3\text{H}]$ glutamate and $[^{14}\text{C}]$ aspartate) were obtained from DuPont Biotechnology Systems. Columns and resins were obtained from Bio-Rad Laboratories. Other enzymes, coenzymes, substrates, chemicals and reagents were obtained from Sigma. The non-fluorescent uncoupling agent 1799 was generously given by Dr. Peter Heytler of the Research Division, E. I. DuPont de Nemours, Wilmington, DE, U.S.A.

Statistics

Values shown are means $\pm$ S.E.M. Unless otherwise indicated, statistical analysis was performed by Student's *t* test for paired and unpaired data. Comparisons of means of *n* groups (*n* > 2) were performed by one-way analysis of variance. *A priori* test was then used to compare means of any two groups, F tests being used as test of significance. *P* < 0.05 was taken to be statistically significant.

RESULTS

2-OGDH sensitivity to pH and Ca^{2+} in permeabilized kidney and liver mitochondria

To determine pH- and Ca^{2+} -sensitivity, kinetic parameters (apparent K_m and $V_{\max}$) were determined at pH 7.1 and 7.7 and as a function of free $[\text{Ca}^{2+}]$ in the range of 0–11 μM . An Eadie plot of the data is shown in Fig. 1. As expected, the apparent K_m of 2-OG was decreased by H^+ and free Ca^{2+} in both organs (Table 1), but the effect was not additive. The sensitivity of the apparent K_m to free Ca^{2+} disappeared as the pH was decreased from 7.7 to 7.1 and was not different in the two organs. At pH 7.7, the $K_{0.5}$ (Ca^{2+}) for 2-OGDH obtained from a double-reciprocal plot was 0.17 μM in the liver and 0.11 μM in the kidney.

Our $K_{0.5}$ (Ca^{2+}) values for 2-OGDH are consistent with the previously reported $K_{0.5}$ value of 0.15 μM in intact rat heart mitochondria (Wan *et al.*, 1989), but slightly lower than that of Rutter *et al.* (1989), who noted a $K_{0.5}$ value for Ca^{2+} of 0.28 μM using permeabilized pig heart mitochondria and 0.21 μM in pig heart mitochondrial extracts in the presence of ADP. All of these values are substantially lower than those previously reported (McCormack & Denton, 1979; Hansford, 1985).

The most striking finding in the permeabilized mitochondria was that $V_{\max}$ was 3.3 times higher in the kidney than the liver (Table 1). $V_{\max}$ was insensitive to changes in free Ca^{2+} , but was inhibited by H^+ . At pH 7.7 $V_{\max}$ was 121.5 ± 3.5 nmol/min per mg of mitochondrial protein in the kidney (*n* = 4) and 36.8 ± 0.6 nmol/min per mg in the liver (*n* = 4). These maximal

Fig. 1. Eadie plot of 2-OGDH activity in permeabilized liver mitochondria

v represents 2-OGDH activity in nmol/min per mg of mitochondrial protein and *s* is the concentration of 2-OG in mM. The example shown was obtained at pH 7.7, $[\text{Ca}^{2+}] = 1 \mu\text{M}$ and 28 °C.

rates fell significantly (Table 1) at pH 7.1 in both organs, to 80.7 ± 2.0 and 31.7 ± 0.5 nmol/min per mg in the kidney and liver respectively.

2-OGDH sensitivity to pH and Ca^{2+} determined in intact mitochondria

When activity of 2-OGDH was indirectly measured in intact mitochondria by assessing O_2 consumption by liver and kidney mitochondria in the presence of various amounts of 2-OG and excess ADP, no significant pH- or Ca^{2+} -sensitivity could be detected. The apparent K_m values for 2-OG in kidney and liver were 0.2 ± 0.02 and 0.5 ± 0.1 mM respectively; both were independent of pH and Ca^{2+} . $V_{\max}$ was 54.5 ± 1.6 ng atoms of O/min per mg of mitochondrial protein in kidney and 17.9 ± 1.1 in liver. Since ADP itself may lower the K_m of 2-OGDH (McCormack & Denton, 1979), other experiments were devised to independently assess the influence of ADP, H^+ and Ca^{2+} on 2-OGDH activity. These $V_{\max}$ values obtained from measurements of O_2 consumption are valid estimates of flux through 2-OGDH in intact mitochondria, since endogenous respiration in the absence of added 2-OG was very low, and since malonate blocked the citric acid cycle at succinate dehydrogenase. This guaranteed a 1:1 relationship between nmol of 2-OG oxidized and ng-atoms of O consumed. It is noteworthy that the $V_{\max}$ values obtained with intact mitochondria were approximately half those for $V_{\max}$ in permeabilized mitochondria. This seems reasonable, since NAD^+ and CoA availability could not be optimized in intact mitochondria as they were in permeabilized mitochondria. The fact that the observed $V_{\max}$ values in intact mitochondria were a large fraction of the $V_{\max}$ values of permeabilized mitochondria suggests that transport does not play a large role in the control of flux.

To test if 2-OG transport is rate-limiting, it was necessary to monitor independently 2-OG generated within the mitochondria and 2-OG transported into the mitochondria from the external media. In the presence of DL-cycloserine, a transaminase inhibitor that does not penetrate the mitochondrial membrane in the time frame of these experiments (Williamson *et al.*, 1974; Meijer *et al.*, 1975; Marra *et al.*, 1977), $[^3\text{H}]$ glutamate would be transaminated to $[^3\text{H}]$ 2-OG exclusively within the mitochondria, whereas $[^{14}\text{C}]$ 2-OG had to be transported across the mitochondrial membrane before its oxidation. Total 2-OGDH flux was determined in both kidney and liver mitochondria at pH 6.6 and 7.0 in the absence (Fig. 2a) and the presence of 5 mM-ADP (Fig. 2b). Of the four conditions studied, H^+ stimulated 2-OGDH flux only in the kidney and only in the absence of added ADP. However, in the absence of ADP, flux through 2-OGDH in both liver and kidney was increased by Ca^{2+} . In the liver, there was a 63% Ca^{2+} -stimulated increase in 2-OGDH flux at pH 7.0 (*P* < 0.005) and a 54% increase at pH 6.6 (*P* < 0.001). In the kidney, Ca^{2+} -stimulated increases were 77% and 61% at pH 7.0 (*P* < 0.001) and pH 6.6 (*P* < 0.001) respectively. In the presence of ADP (Fig. 2b), 2-OGDH was insensitive to increases in both Ca^{2+} and H^+ in both organs, suggesting that low mitochondrial-matrix ATP/ADP ratios preclude H^+ and Ca^{2+} control. We wish to emphasize that the very low values of this ATP/ADP ratio in these experiments are likely to be unphysiological.

Incubations containing radiolabelled substrates and subsequent separation of the labelled metabolites provided not only a second method to determine 2-OGDH flux but also the means to track the source of the 2-OG. In these experiments, 2-OGDH flux was calculated from the sum of radioisotopes appearing in the oxidation products, after correction for metabolic losses of $^{14}\text{CO}_2$ and $^3\text{H}_2\text{O}$. 2-OGDH flux determined in this manner was within the confidence limits of the total 2-OGDH flux determined from changes in assayed aspartate and 2-OG. If 2-OG membrane

Table 1. Effect of Ca^{2+} and pH on the apparent K_m and $V_{\max}$ of 2-OGDH in permeabilized mitochondria

The apparent K_m (mM) and $V_{\max}$ (nmol/min per mg) of 2-OGDH in permeabilized mitochondria was obtained from Eadie plots similar to that shown in Fig. 1, except that pH of the buffer and the intramitochondrial free $[\text{Ca}^{2+}]$ were varied as shown. Data shown are means $\pm$ S.E.M. ($n = 4$) for each condition. At pH 7.1, the apparent K_m did not significantly change as the Ca^{2+} concentration varied and was not different in the two organs. However, $V_{\max}$ was significantly higher in kidney than in liver ($P < 0.001$ by paired t test). By analysis of variance, $V_{\max}$ was insensitive to changing $[\text{Ca}^{2+}]$ at both pH 7.7 and 7.1 in both liver ($P = 0.4318$ and $P = 0.9524$ at pH 7.7 and 7.1 respectively) and kidney ($P = 0.4318$ and $P = 0.0624$). $V_{\max}$ was inhibited by increasing H^+ concentration in both liver ($P < 0.0001$ by paired t test) and kidney ($P < 0.0002$).

$[\text{Ca}^{2+}]$ (μM)	K_m				$V_{\max}$			
	Liver		Kidney		Liver		Kidney	
	pH 7.1	pH 7.7	pH 7.1	pH 7.7	pH 7.1	pH 7.7	pH 7.1	pH 7.7
0	0.14 ± 0.01	2.98 ± 0.20	0.15 ± 0.02	4.32 ± 0.59	31.22 ± 1.80	35.98 ± 1.46	69.59 ± 2.91	130.69 ± 5.04
0.10	0.15 ± 0.01	1.84 ± 0.26	0.18 ± 0.01	2.35 ± 0.12	33.37 ± 2.72	39.29 ± 4.35	82.19 ± 2.38	112.47 ± 9.02
0.25	0.17 ± 0.01	1.18 ± 0.09	0.17 ± 0.01	1.87 ± 0.08	32.24 ± 1.91	38.31 ± 3.26	81.57 ± 2.66	116.84 ± 4.50
0.50	0.14 ± 0.01	0.50 ± 0.03	0.15 ± 0.01	1.02 ± 0.13	31.96 ± 2.70	36.49 ± 2.58	83.35 ± 4.18	132.31 ± 11.75
1.00	0.13 ± 0.01	0.29 ± 0.02	0.16 ± 0.02	0.59 ± 0.08	31.91 ± 2.68	36.08 ± 2.36	83.66 ± 1.96	134.63 ± 7.72
11.0	0.10 ± 0.01	0.23 ± 0.01	0.14 ± 0.01	0.23 ± 0.01	29.42 ± 2.76	34.68 ± 2.34	83.56 ± 3.13	131.94 ± 5.26

Fig. 2. Effect of pH and matrix free $[\text{Ca}^{2+}]$ on 2-OGDH flux in intact mitochondria

Liver and kidney mitochondria were incubated in medium B at pH 6.6 or pH 7.0 with intramitochondrial Ca^{2+} concentrations adjusted to 0.03 μM and 0.5 μM in the absence of ADP (State 4; a) and in the presence of 5 mM-ADP (State 3; b) at 28 °C. Radiolabelled substrates were 0.5 mM- $[\text{C}^{14}]$ 2-OG and 1 mM- $[\text{H}^3]$ glutamate: neither concentration was saturating, so that Ca^{2+} - or H^+ -induced changes in the apparent K_m of 2-OGDH would be observed as an increase in flux. Total 2-OGDH flux ($\square$) was calculated from changes in assayed aspartate and 2-OG as described in the Methods section, in both State 3 ($n = 7$ in liver, $n = 4$ in kidney) and State 4 ($n = 6$ in liver, $n = 5$ in kidney). $[\text{H}^3]$ 2-OGDH flux ($\blacksquare$) was determined from the rate of accumulation of ^3H in malate and succinate in samples taken at 0, 2 and 4 min in both State 3 ($n = 5$, liver; $n = 3$, kidney) and State 4 ($n = 4$, both).

transport were the rate-limiting factor accounting for the different responses in liver and kidney, then one would expect to see that the oxidation of $[\text{H}^3]$ 2-OG generated within the mitochondria would follow the same pattern as 2-OGDH in permeabilized mitochondria. This was not the case. In both liver and kidney, oxidation of 2-OG generated inside the mitochondria did not increase with decreasing pH (Fig. 2). Indeed, increased H^+ concentration tended to inhibit oxidation of $[\text{H}^3]$ 2-OG.

Fig. 3. Turnover of the 2-OG pool

Liver and kidney mitochondria were incubated in buffer B (see the Methods section) with $[\text{Ca}^{2+}] = 0.5 \mu\text{M}$, pH 7.0, malate 1 mM. $\bullet$, Disappearance of $[\text{C}^{14}]$ 2-OG from the medium. $\circ$, Appearance in the medium of $[\text{H}^3]$ 2-OG derived from conversion of $[\text{H}^3]$ glutamate. In liver mitochondria (a) the pool of total 2-OG remained constant, whereas in kidney mitochondria (b) the total pool of 2-OG decreased precipitously during the experiment.

Transport of 2-OG into intact mitochondria

A minimum value for transport of 2-OG into the mitochondria was determined from the radio-isotopic turnover of the 2-OG

Table 2. Effect of Ca^{2+} and pH on $[^{14}\text{C}]2\text{-OG}$ transport into the mitochondria

Liver and kidney mitochondria were incubated as described in Fig. 2 legend. The minimal rate of $[^{14}\text{C}]2\text{-OG}$ entry was determined from measurements of $[^{14}\text{C}]2\text{-OG}$ disappearance from the medium as described in the Methods section. Entry was not significantly changed by either pH or Ca^{2+} , although in the State-4 kidney entry at pH 7.0 and $0.03\ \mu\text{M}\text{-Ca}^{2+}$ was significantly lower ($P < 0.05$). For each experiment, the 2 and 4 min values, which were not significantly different, were averaged. Values shown are means $\pm$ S.E.M.: in State 4, $n = 4$ for liver and kidney; in State 3, $n = 5$ for liver and $n = 3$ for kidney.

$[\text{Ca}^{2+}]$ (μM)	pH	$[^{14}\text{C}]2\text{-OG}$ transport (nmol/min per mg)			
		Liver		Kidney	
		State 4	State 3	State 4	State 3
0.03	6.6	12.37	11.29	13.82	37.88
		± 0.87	± 1.28	± 3.55	± 5.09
	7.0	12.05	16.47	5.22	28.40
0.5	6.6	± 2.00	± 3.35	± 1.19	± 2.32
		13.53	14.02	16.94	29.94
	7.0	± 0.89	± 2.33	± 2.10	± 4.98
		13.34	11.89	12.25	23.76
		± 0.92	± 3.12	± 1.41	± 1.32

Fig. 4. Effect of pH and Ca^{2+} on the rate of formation of $[^{14}\text{C}]$ glutamate from $[^{14}\text{C}]2\text{-OG}$

Liver and kidney mitochondria were incubated as described in Fig. 2, and the rate of formation of $[^{14}\text{C}]$ glutamate was determined as described in the Methods section. In State 4 (a), $n = 4$. In State 3 (b), $n = 5$ for liver and $n = 3$ for kidney.

pool (Fig. 3). Disappearance of $[^{14}\text{C}]2\text{-OG}$ over time was approximately linear (Fig. 3), providing a minimal estimate of transport into both liver and kidney mitochondria. The estimated rate of 2-OG transport is necessarily a lower limit, since $2\text{-OG}_{\text{in}}/2\text{-OG}_{\text{out}}$ exchange is active, as indicated by appearance of $[^3\text{H}]2\text{-OG}$ derived from $[^3\text{H}]$ glutamate. More accurate determinations of 2-OG transport are difficult in the absence of knowledge of 2-OG exchange rates. The effects of Ca^{2+} , pH and ADP on minimal rates of 2-OG entry into the mitochondria are shown in Table 2. Transport of 2-OG was sufficiently rapid to support total 2-OGDH flux (Fig. 2), in both liver and kidney mitochondria incubated under State-4 conditions. Thus transport is not rate-limiting for 2-OG oxidation in State 4. In State 3, transport provides at least as much intramitochondrial 2-OG in the kidney as in the liver. Therefore differences in H^+ -sensitivities of 2-OGDH flux (Fig. 2) in the two organs do not reside in the transporter.

Our minimum estimate of 2-OG transport is higher than the rate of formation of ^{14}C -labelled products of 2-OG, because some of the $[^{14}\text{C}]2\text{-OG}$ which enters the mitochondria undergoes reverse transamination and appears as $[^{14}\text{C}]$ glutamate. The formation of $[^{14}\text{C}]$ glutamate from $[^{14}\text{C}]2\text{-OG}$ by reverse transamination was evaluated (Fig. 4). Under all conditions studied, $[^{14}\text{C}]2\text{-OG}$ was converted more rapidly into $[^{14}\text{C}]$ glutamate in the liver than in the kidney. This reverse transamination is dependent on the amount of transaminase in the mitochondria and on the

activity of the glutamate-hydroxyl carrier (Schoolwerth *et al.*, 1983). The glutamate/aspartate exchanger does not catalyse glutamate/glutamate exchange, but the glutamate-hydroxyl transporter does. Its activity has been previously studied in both liver and kidney, and the rates of formation of $[^{14}\text{C}]$ glutamate from $[^{14}\text{C}]2\text{-OG}$ are compatible with previously measured activity of glutamate/glutamate exchange on the glutamate-hydroxyl carrier (Schoolwerth *et al.*, 1978; Kunin & Tannen, 1979). On the other hand, transaminase activity is many orders of magnitude higher than glutamate/glutamate exchange, i.e. V_{max} of GOT is approx. $1.7\ \mu\text{mol}/\text{min per mg}$ in the permeabilized kidney and $2.7\ \mu\text{mol}/\text{min per mg}$ in permeabilized liver (B. C. Smith & K. F. LaNoue, unpublished work).

In the liver, 2-OGDH used 2-OG generated within the mitochondria in preference to 2-OG transported in (Fig. 2). Thus most of the total flux was derived from $[^3\text{H}]2\text{-OG}$ in the liver, whereas in the kidney $[^3\text{H}]2\text{-OG}$ flux represented a much smaller portion of the total flux.

To assess further whether 2-OGDH preferentially utilizes 2-OG generated from intramitochondrial transamination, the fractional ^3H specific radioactivity of products of 2-OGDH (malate and succinate) was compared with the fraction of intramitochondrial 2-OG generated from $[2,3\text{-}^3\text{H}]$ glutamate (Table 3). The rat liver mitochondria show a clear preference for the 2-OG produced by transamination, since the fractional ^3H specific radioactivity of the products is significantly higher than

Table 3. Fraction of products of 2-OGDH derived from [³H]glutamate compared with the fraction of intramitochondrial 2-OG derived from [³H]glutamate

The fraction of products of 2-OGDH derived from [³H]glutamate was determined by dividing the 'corrected' ³H specific radioactivity of succinate and malate by the ³H specific radioactivity of the [2,3-³H]glutamate added to the incubation mixture. The fraction of the substrate for 2-OGDH (intramitochondrial 2-OG) derived from [2,3-³H]glutamate was estimated by dividing the rate of generation of intramitochondrial [³H]2-OG by the sum of [³H]2-OG generation plus [¹⁴C]2-OG entry into the mitochondria. Since [¹⁴C]2-OG exchange rate could not be determined, [¹⁴C]2-OG entry rate is a minimal value, and the estimate of the fractional ³H specific radioactivity of matrix 2-OG was a maximal value. Values shown are means $\pm$ S.E.M. For each experimental condition, the 2 and 4 min values of malate and succinate were not significantly different and were included in the mean. For each experimental condition, in State 4 $n = 4$, and in State 3 $n = 5$ for liver and $n = 3$ for kidney. * $P < 0.05$, product versus substrate.

Tissue	[Ca ²⁺] (μ M)	pH	State 4		State 3	
			Intra-mitochondrial 2-OG	Products of 2-OGDH	Intra-mitochondrial 2-OG	Products of 2-OGDH
Liver	0.03	6.6	0.183	0.924*	0.639	0.802
			± 0.025	± 0.062	± 0.053	± 0.052
		7.0	0.237	0.821*	0.633	0.707
			± 0.045	± 0.048	± 0.040	± 0.027
	0.50	6.6	0.228	0.902*	0.611	0.824*
			± 0.014	± 0.033	± 0.057	± 0.034
		7.0	0.326	0.840*	0.592	0.800*
			± 0.050	± 0.064	± 0.074	± 0.048
Kidney	0.03	6.6	0.079	0.292	0.104	0.159
			± 0.011	± 0.082	± 0.020	± 0.015
		7.0	0.354	0.464	0.190	0.255
			± 0.064	± 0.054	± 0.034	± 0.045
	0.50	6.6	0.099	0.233	0.168	0.261
			± 0.015	± 0.017	± 0.031	± 0.054
		7.0	0.147	0.254	0.337	0.404
			± 0.017	± 0.014	± 0.032	± 0.055

Fig. 5. Effect of pH and Ca²⁺ on the rate of production of intramitochondrial 2-OG by transamination of glutamate

Liver and kidney mitochondria were incubated as described in Fig. 2. The formation of 2-OG from the transamination of glutamate was determined from the rate of formation of aspartate in the samples. (a) State 4, $n = 4$, (b) State 3, $n = 5$, liver; $n = 3$, kidney.

the maximal fractional ³H specific radioactivity of the substrate. A similar trend was present in the kidney, but differences did not reach statistical significance.

The effects of pH on GOT and the aspartate/glutamate carrier were monitored by measurement of change in aspartate production (Fig. 5). H⁺ inhibited the GOT-aspartate/glutamate carrier complex in both liver and kidney.

DISCUSSION

The mitochondrial enzyme 2-OGDH plays an important role in acid-base balance via its control over 2-OG levels and thus ammoniagenesis. In previously reported studies of the isolated enzyme in a variety of organs, 2-OGDH activity was modulated by both Ca²⁺ and pH (McCormack & Denton, 1979; Lowry & Ross, 1980; Rutter & Denton, 1988). In the kidney, 2-OG levels

decrease, and ammonia production and gluconeogenesis are stimulated, in response to increases in free cytosolic Ca²⁺ brought about by Ca²⁺-mobilizing hormones, as well as by increases in H⁺ (Kamm *et al.*, 1967; Kurokawa & Massry, 1973; Schoolwerth & LaNoue, 1983). In the liver, 2-OG levels fall in response to hormonal mobilization of intracellular Ca²⁺, and gluconeogenesis increases (Sterniczuk *et al.*, 1991), but H⁺ does not produce a similar stimulation. To elucidate the molecular mechanism of 2-OGDH regulation by Ca²⁺ and H⁺ ions, we systematically studied Ca²⁺- and pH-sensitivities of kidney and liver 2-OGDH in permeabilized and intact mitochondria. In permeabilized liver and kidney mitochondria in which the native microenvironment is altered, 2-OGDH exhibited sensitivity to both pH and Ca²⁺ (Table 1). The major difference was that V_{max} was more than 3 times greater in the kidney than the liver (Table 1). Therefore differences in intrinsic properties of the enzyme in the two organs

could not account for the differing responses to acidosis. In contrast, in intact mitochondria, in which the topographical arrangement and biochemical domains surrounding 2-OGDH were not perturbed, significant differences were observed between the liver and kidney enzymes. Thus, although both were stimulated by Ca^{2+} , decreases in pH stimulated only the kidney enzyme, but inhibited liver 2-OGDH (Fig. 2a). In addition, liver 2-OGDH preferentially utilized 2-OG derived from transamination, whereas the kidney enzyme relied more on 2-OG transport for its substrate supply (Fig. 2, Table 3). The differences between permeabilized and intact mitochondria were not due to limited access of the enzyme in intact mitochondria to external 2-OG, since 2-OG transport was not rate-limiting (Table 2). If transport had been limiting, then the oxidation of 2-OG generated within the mitochondria would exhibit similar Ca^{2+} - and pH-sensitivities to the enzyme in the permeabilized mitochondria, and this was not the case (Fig. 2). The marked preference of liver 2-OGDH for 2-OG derived from exogenous glutamate (Fig. 2, Table 3) implies that 2-OG generated by transamination has preferential access to the active site of 2-OGDH, especially in liver. This strongly suggests a degree of micro-compartmentation (or channelling) between 2-OGDH and the transaminase.

Micro-compartmentation has been defined as a situation in which a metabolite moves from its synthesizing enzyme to another enzyme without mixing with the total substrate pool in the macro-compartment (Schoolwerth & LaNoue, 1980). In essence, substrates are channelled from one enzyme to a second enzyme, or from a carrier to an enzyme or vice versa. There are glutamate micro-compartments in the kidney which permit glutamate entering the mitochondria on the glutamate-aspartate carrier to be transaminated without mixing with intra-mitochondrial glutamate, which is deaminated to 2-OG by glutamate dehydrogenase (Schoolwerth & LaNoue, 1980). In these experiments (Schoolwerth & LaNoue, 1980) the specific radioactivities of matrix glutamate and formed CO_2 were measured in radioisotopic experiments using [^{14}C]glutamine and unlabelled glutamate in physiological concentrations. Despite the fact that matrix glutamate was highly ^{14}C -labelled, the products of glutamate oxidation tended to be unlabelled when transaminase activity was high, but to be ^{14}C -labelled when dehydrogenase activity was high.

There have also been reports of micro-compartmentation and channelling between GOT and the glutamate/aspartate transporter in the liver (Duszynski *et al.*, 1978). Radioisotopic data suggested that aspartate formed from transamination is channelled from GOT directly to the glutamate-aspartate carrier without mixing with matrix aspartate. Also in the liver, investigators have noted channelling of urea-cycle intermediates (Cheung *et al.*, 1989). In these studies of α -toxin-permeabilized hepatocytes using [^{14}C]bicarbonate to label and differentiate substrates produced within the cycle from those added outside the cycle, channelling was demonstrated between argininosuccinate lyase and arginase, of argininosuccinate between its synthetase and lyase, and of citrulline from its site of synthesis by ornithine transcarbamoylase within the mitochondrial matrix to the cytosolic enzyme argininosuccinate synthetase. For arginine channelling was very tight, but channelling of the other two substrates was less so. Numerous other examples of channelling in a variety of cell types have been reported (for review see Srere, 1987).

Further support for the concept of channelling is provided by studies of the interaction between 2-OGDH and GOT. Fahien *et al.* (1988) have shown that, under certain circumstances, a binary complex between these two enzymes can be demonstrated. The purified enzymes either alone or together were incubated in poly(ethylene glycol). After a short incubation period, they were

centrifuged, sedimenting precipitated enzyme complexes. Both the resuspended pellet of complexed enzymes and the supernatant containing uncomplexed enzymes were assayed for enzyme activity. In studies using equal concentrations of 2-OGDH and GOT (0.1 mg/ml), in the presence of poly(ethylene glycol) 78 % of 2-OGDH was complexed in Tris/EDTA buffer and 71 % in potassium phosphate/EDTA buffer. However, it is somewhat difficult to extrapolate these studies *in vitro* using poly(ethylene glycol) to the situation *in vivo*, where a different ionic concentration or other intramitochondrial factors may affect protein interactions and complexation.

In view of the finding of the formation of enzyme-enzyme complexes between 2-OGDH and GOT, as well as the present studies demonstrating preferential access of 2-OG generated by transamination to 2-OGDH, we propose that channelling between the two enzymes occurs in both organs. However, because there is less 2-OGDH and more GOT in liver than in kidney, it would appear reasonable that a much greater percentage of the 2-OGDH is complexed to the transaminase in the liver than in the kidney. Thus one would expect that the free 2-OGDH in the kidney would exhibit the H^+ - and Ca^{2+} -sensitivity of the isolated enzyme, whereas the complexed 2-OGDH would be more likely to exhibit the pH-sensitivity of the transaminase and the glutamate/aspartate carrier (Fig. 5). Therefore we propose that differences in the ratio of the two enzymes within the two organs (liver and kidney) could account for the noted differences in 2-OGDH sensitivities. To our knowledge, this is the first report of a significant physiological role for micro-compartmentation.

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