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Subcellular distribution of malate–aspartate cycle intermediates during normoxia and anoxia in the heart

Rudolf J. Wiesner [a], Ulrike Kreutzer [a], Peter Rösen [b] and Manfred K. Grieshaber [a]

[a] *Institut für Zoologie, Lehrstuhl für Tierphysiologie and* [b] *Diabetes Forschungsinstitut, University of Düsseldorf, Düsseldorf (F.R.G.)*

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The subcellular distribution of adenine nucleotides, phosphocreatine and intermediates of the malate–aspartate cycle was investigated in adult rat heart myocytes under normoxia and anoxia. Cytosolic and mitochondrial concentrations of metabolites were determined by a fractionation method using digitonin. Under normoxia, cytosolic/mitochondrial gradients were found for ATP ($c/m = 4$), AMP ($c/m < 0.01$), citrate ($c/m = 0.5$), aspartate ($c/m = 3$), glutamate ($c/m = 2$), while phosphocreatine and glutamine were confined to the cytosolic space. No gradients were found for malate and 2-oxoglutarate. The results show that the transport of electrons from the cytosol into the mitochondria is supported by the glutamate gradient and by a high glutamate/aspartate ratio inside the mitochondria (Glu/Asp = 15) which is maintained by the energy-dependent Glu–Asp exchange across the mitochondrial membrane. Under anoxia, cytosolic glutamate is transaminated with pyruvate, yielding alanine and 2-oxoglutarate, which is oxidized to succinate inside the mitochondria and leaves the cell. The data indicate that stimulation of transamination is caused by a mass action effect following a decrease in cytosolic 2-oxoglutarate which may be due to succinate–2-oxoglutarate exchange across the mitochondrial membrane. Inhibition of the energy-dependent inward transport of glutamate may support this process.

Introduction

Some amino and carboxylic acids play a crucial role in intermediary metabolism in the heart. Malate, oxaloacetate and 2-oxoglutarate are involved in the Krebs cycle and, together with glutamate and aspartate, in the transport of electrons from the cytosolic into the mitochondrial compartment, i.e., the malate–aspartate cycle [1]. This transfer is essential for the maintenance of energy metabolism when the heart is oxidizing glucose and lactate, which cover approx. 60% of the substrates used [2]. Our present knowledge about the regulation of this cycle and its interaction with the Krebs cycle is derived mainly from studies on isolated mitochondria and enzymes (for review, see Ref. 3). Information about the cytosolic-mitochondrial distribution of its intermediates in the heart muscle cell is rare, although it is a prerequisite for understanding regulation phenomena in situ [4].

There is now a lot of evidence that isolated, Ca^{2+}-tolerant cardiomyocytes are suitable as an

Correspondence: R.J. Wiesner, Institut für Zoologie IV, Lehrstuhl für Tierphysiologie, Geb. 26.12.00, Universitätsstr. 1, 4000 Düsseldorf 1, F.R.G.

experimental model for studying the metabolism of the heart [5–7]. This system combined with the rapid separation of the mitochondrial from the cytosolic compartment using digitonin [8] has been successfully used for the determination of the compartmentation of adenine nucleotides and their degradation products in the heart [9].

We used isolated cardiomyocytes together with the digitonin fractionation method to reveal the compartmentation of metabolites involved in energy metabolism and in the malate–aspartate cycle under steady-state conditions and alterations of the subcellular distribution of these metabolites following anoxia. A preliminary study was published previously [10].

Methods

Preparation and incubation

Ca^{2+}-tolerant myocytes were prepared from hearts of adult, male Wistar rats by Langendorff perfusion with crude collagenase (for a detailed description see Ref. 6). The preparations used in this study contained 87 ± 3% of cells with the elongated, rod-shaped morphology of myocytes in situ, examined by light microscopy ($n = 27$). Cells were incubated in a gyratory water-bath shaker at 37° C in a physiological, phosphate-buffered saline (pH 7.4) containing NaCl (121 mM), KCl (5 mM), $MgSO_4$ (1.2 mM), Na_2HPO_4 (16 mM), K_2HPO_4 (1.2 mM), glucose (11 mM), taurine (60 mM), creatine (23 mM), glutamine (0.7 mM), bovine serum albumin (2%; Fraction V, Boehringer) and a complete mixture of amino acids and vitamins [6].

Oxygenated cells were incubated in open flasks. Anoxic cells were incubated in stoppered flasks flushed with humidified argon for 5 min. Under both conditions, the percentage of rod-shaped cells did not change significantly during 1 h of incubation. The cell titer was approx. 375 000 cells/ml, equal to approx. 2 mg of cellular protein/ml.

Extraction and fractionation of cells

Cells were simultaneously separated from the incubation medium and extracted in perchloric acid, as described in detail by Geisbuhler et al. [9]. In short, the suspension was injected into buffered sucrose solution (0.25 M), layered on bromododecane, which was layered on perchloric acid. Separation and extraction was achieved by centrifugation of the cells through the bromododecane into perchloric acid.

Mitochondria were selectively extracted by adding digitonin (0.6 mg/ml) to the sucrose buffer, an agent which disrupts the sarcolemma, but leaves the mitochondrial membrane intact [8]. In order to avoid alterations at ATP levels, atractyloside (0.24 mg/ml) and oligomycin (0.02 mg/ml) were also present [9].

Determination of intracellular water spaces

In order to allow the calculation of cytosolic and mitochondrial metabolite concentrations, it was necessary to determine the water content of the subcellular spaces. Therefore, cells were incubated with ^3H_2O (5 μCi/ml) and incubated as described above. [^{14}C]Inulin (1 μCi/ml) was present to permit correction for extracellular water, contaminating the extracts. Samples of the incubation medium and of the cellular and mitochondrial extracts, respectively, were combusted (Packard Oxidizer, Mod. 306) and the resulting ^3H_2O and $^{14}CO_2$ were counted separately in a liquid scintillation counter. The cellular and mitochondrial water spaces, respectively, were calculated according to the equation:

$$\text{water space (ml)} = \frac{(\text{dpm } ^3\text{H total})_{\text{extract}}}{(\text{dpm } ^3\text{H/ml})_{\text{medium}}} - \frac{(\text{dpm } ^{14}\text{C total})_{\text{extract}}}{(\text{dpm } ^{14}\text{C/ml})_{\text{medium}}}$$

Determination of metabolites and enzyme activities

Extracts and deproteinized incubation media were neutralized using K_2CO_3 (2 M) in triethanolamine (0.5 M) [9]. ATP, phosphocreatine, citrate, malate, 2-oxoglutarate, succinate and lactate as well as lactate dehydrogenase and citrate synthase were measured by standard enzymatic methods [11], using double-wavelength spectrophotometry (ZWS 11, Sigma) or fluorometry (Foci A4, Farrand). Amino acids were measured using an automated amino acid analyzer (Liquimat III, Kontron) with ninhydrin derivatization.

Total cellular enzymatic activities were estimated by dilution of a sample of the cell suspen-

sion with distilled water (1/1, v/v) followed by ultrasonic disruption. Release of cytosolic and mitochondrial compounds during incubation with digitonin was estimated by measuring release of the marker enzymes, lactate dehydrogenase and citrate synthase, respectively, into the digitonin-containing buffer.

Total cellular protein was estimated after dissolving the perchloric acid pellet in NaOH (1 M) by the Pierce BCA method [12] using bovine serum albumin as standard.

Metabolite and water contents were calculated on the basis of total cellular protein (nmol/mg protein). Cytosolic contents were calculated by subtracting the digitonin-insoluble content (equivalent to the mitochondrial content) from the total cellular content. Cytosolic and mitochondrial concentrations of metabolites in anoxic cells were compared with the corresponding value obtained in oxygenated cells incubated for the same time period using the non-parametric, paired *U*-test of Wilcoxon, Mann and Whitney [13] and differences with $P < 0.05$ were assumed to be significant.

Results

Release of metabolic end-products (Table I)

In oxygenated cells, only small amounts of lactate were produced with a rate of approx. 8 nmol/mg protein per min, whereas no succinate was released; both findings indicate that anaerobic metabolism was negligible under these conditions.

During anoxia, anaerobic glycolysis was accelerated instantaneously. The rate of lactate production was linear over the entire incubation period and amounted to 60 nmol/mg protein per min, which is equivalent to the maximum rate reported for the anoxic rat heart (10 μmol/g wet wt. per min [14]). 15 min after induction of anoxia, significant amounts of succinate were released into the incubation medium. These data substantiate the transition of energy metabolism to anaerobic mechanisms under the experimental conditions used.

Release of alanine occurred under both conditions, but was significantly higher during anoxia.

Validation of the fractionation method

During treatment with digitonin as described in methods, 97 ± 10% ($n = 7$) of total cellular L-lactate dehydrogenase, the cytosolic marker enzyme, were released, indicating that disruption of the sarcolemmal membrane was complete. At the same time, less than 1% of total cellular citrate synthase activity was released, indicating that mitochondria remained intact.

Subcellular water spaces

Using ^3H_2O, the cellular water content was found to be 3.7 ± 1.2 μl/mg protein, the mitochondrial water content was 1.3 ± 0.5 μl/mg protein yielding 2.4 ± 0.8 μl/mg protein for the cytosolic space. These values did not change during incubation, neither under oxygenated nor under anoxic conditions, and were used to convert metabolite contents of the subcellular spaces (nmol/mg protein) into concentrations (mmol/liter).

TABLE I

METABOLIC END-PRODUCT RELEASE

Release of lactate, succinate and alanine into the incubation medium by isolated rat heart cells under oxygenated and anoxic conditions (nmol/mg protein; mean ± S.D.; $n = 6$–10). Values obtained in anoxic cells were compared to the corresponding value in oxygenated cells incubated for the same time period using the paired Wilcoxon *U*-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

	Incubation time (min)							
	oxygenated cells				anoxic cells			
	5	15	30	60	5	15	30	60
Lactate	67 ± 39	86 ± 30	360 ± 180	486 ± 168	199 ± 74 **	837 ± 222 ***	1895 ± 303 ***	3631 ± 475 **
Succinate	n.d.	n.d.	n.d.	n.d.	n.d.	2.9 ± 0.9 **	10.5 ± 2.6 **	14.2 ± 4.6 **
Alanine	0.5 ± 2.4	5.9 ± 1.7	9.0 ± 3.4	17.4 ± 3.5	5.3 ± 2.3 **	9.0 ± 3.7 *	13.3 ± 3.7 *	22.4 ± 6.7 *

n.d., not detectable.

TABLE II

COMPARTMENTATION OF ADENINE NUCLEOTIDES AND PHOSPHOCREATINE

Concentration of adenine nucleotides and phosphocreatine in the cytosolic and mitochondrial compartment of isolated rat heart cells under oxygenated and anoxic conditions (mmol/liter; mean ± S.D.; $n = 6$ or 7). Values obtained in anoxic cells were compared with the corresponding value in oxygenated cells incubated for the same time period using the Wilcoxon U-test: * $P < 0.05$.

	Incubation time (min)							
	oxygenated cells				anoxic cells			
	5	15	30	60	5	15	30	60
ATP								
Cyto	8.81 ± 0.72	9.00 ± 0.45	8.89 ± 1.65	8.68 ± 0.66	8.85 ± 0.41	8.56 ± 1.07	7.57 ± 1.81	7.33 ± 1.03
Mito	2.08 ± 0.24	2.16 ± 0.32	2.08 ± 0.24	1.84 ± 0.40	2.16 ± 0.32	2.32 ± 0.48	2.40 ± 0.32	2.32 ± 0.32
ADP								
Cyto	0.78 ± 0.21	0.62 ± 0.45	0.58 ± 0.25	0.54 ± 0.16	0.78 ± 0.33	1.07 ± 0.45	0.70 ± 0.45	0.70 ± 0.21
Mito	1.76 ± 0.24	2.16 ± 0.40	2.24 ± 0.48	1.76 ± 0.32	1.76 ± 0.40	1.76 ± 0.32	2.00 ± 0.56	2.00 ± 0.16
AMP								
Cyto	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mito	0.80 ± 0.48	0.80 ± 0.32	1.12 ± 0.32	0.80 ± 0.04	0.96 ± 0.24	1.12 ± 0.04	0.96 ± 0.24	0.72 ± 0.24
PC								
Cyto	30.90 ± 2.84	28.11 ± 2.88	25.27 ± 4.37	24.28 ± 4.53	26.90 ± 3.10	22.35 ± 4.16	17.57 ± 4.40	15.27 ± 3.46 *
Mito	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detectable.

Compartmentation of adenine nucleotides and phosphocreatine (Table II)

The cytosolic concentration of ATP amounted to 8 mmol/liter and did not change significantly during incubation. The mitochondrial concentration remained at a level of approx. 2 mmol/liter.

The concentrations of ADP were in the range of 0.6–0.8 mmol/liter in the cytosol and around 2 mmol/liter in the mitochondria. It has to be taken into account, however, that most of the cellular ADP is bound to the myofibrils [15], which are probably sedimented and extracted after treatment of cells with digitonin. Therefore, the values measured certainly do not represent the free concentrations of ADP in the compartments.

The results further revealed that AMP is present in the cytosolic space in very low concentrations (detection limit of the methods used: 0.01 mmol/liter), whereas the mitochondrial concentration was in the range of 1 mmol/liter.

Phosphocreatine was found exclusively in the cytosol at a concentration of approx. 25 mmol/liter. During 1 h of anoxic incubation, this value decreased to 15 mmol/liter.

Compartmentation of amino acids related to the Krebs cycle (Table III)

Under normoxia, cytosolic as well as mitochondrial concentrations of the amino acids remained stable for at least 30 min. Thereafter, cytosolic aspartate and glutamine slightly decreased. In general, the cytosolic concentrations of the amino acids were higher than the mitochondrial concentrations. Glutamine was found to be confined to the cytosol, whereas alanine was present in both compartments, the cytosolic concentration exceeding the mitochondrial by a factor of 4. The cytosolic/mitochondrial concentration ratio for aspartate was about 3 and for glutamate about 2.

During anoxia, the cytosolic aspartate concentration initially increased, but fell to very low levels after 1 h, whereas the mitochondrial concentration remained at a constant level of about 0.25 mmol/liter. Anoxia led to a rapid decrease of glutamate in both compartments. After 1 h, the mitochondrial concentration was higher than the cytosolic concentration. Cytosolic alanine rose significantly, whereas the increase in the mitochondrial compartment was of smaller magnitude.

TABLE III

COMPARTMENTATION OF AMINO ACIDS

Concentration of aspartate, glutamate, glutamine and alanine in the cytosolic and mitochondrial compartment of isolated rat heart cells under oxygenated and anoxic conditions (mmol/liter; mean ± S.D.; $n = 6$ or 7). Statistical evaluation of the data, see Table I.

	Incubation time (min)							
	oxygenated cells				anoxic cells			
	5	15	30	60	5	15	30	60
Asp								
Cyto	0.67 ± 0.36	0.58 ± 0.12	0.79 ± 0.29	0.29 ± 0.12	0.94 ± 0.21	1.10 ± 0.27 **	0.55 ± 0.24	0.17 ± 0.15
Mito	0.23 ± 0.07	0.23 ± 0.09	0.25 ± 0.14	0.22 ± 0.09	0.24 ± 0.07	0.18 ± 0.09	0.30 ± 0.18	0.22 ± 0.15
Glu								
Cyto	7.00 ± 1.46	6.61 ± 2.75	8.44 ± 2.03	5.79 ± 1.12	5.28 ± 0.93 *	2.19 ± 0.84 **	0.95 ± 0.41 **	0.92 ± 0.77 **
Mito	3.47 ± 0.42	3.51 ± 0.66	3.84 ± 0.90	3.01 ± 0.70	2.19 ± 0.47 **	1.78 ± 0.51 **	2.02 ± 1.33 *	1.46 ± 0.84 *
Gln								
Cyto	4.91 ± 1.75	4.36 ± 1.13	3.81 ± 1.65	2.65 ± 0.80	4.96 ± 1.50	4.64 ± 1.71	4.00 ± 0.41	2.94 ± 1.81
Mito	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ala								
Cyto	2.76 ± 1.03	3.28 ± 1.56	3.09 ± 1.23	3.04 ± 1.35	5.12 ± 1.39 *	5.85 ± 1.66 *	4.98 ± 2.12	5.42 ± 2.63
Mito	0.81 ± 0.41	0.79 ± 0.43	0.65 ± 0.32	0.90 ± 0.27	0.92 ± 0.29	1.17 ± 0.47	1.35 ± 0.67	1.43 ± 0.48

n.d., not detectable.

TABLE IV

COMPARTMENTATION OF CARBOXYLIC ACIDS

Concentration of malate, 2-oxoglutarate (2-OG), citrate and pyruvate in the cytosolic and mitochondrial compartment of isolated rat heart cells under oxygenated and anoxic conditions (mmol/liter; mean ± S.D.; $n = 6$ or 7). Statistical evaluation of the data see Table I.

	Incubation time (min)							
	oxygenated cells				anoxic cells			
	5	15	30	60	5	15	30	60
Malate								
Cyto	0.18 ± 0.06	0.22 ± 0.12	0.30 ± 0.09	0.43 ± 0.13	0.82 ± 0.21 **	2.00 ± 0.56 **	2.42 ± 0.53 **	1.38 ± 0.38 **
Mito	0.26 ± 0.06	0.29 ± 0.09	0.45 ± 0.18	0.38 ± 0.13	0.48 ± 0.19 *	0.86 ± 0.22 **	1.09 ± 0.22 **	0.58 ± 0.14 *
2-OG								
Cyto	0.25 ± 0.11	0.20 ± 0.08	0.18 ± 0.07	0.17 ± 0.08	0.29 ± 0.09	0.11 ± 0.04 *	0.06 ± 0.04 *	0.05 ± 0.02 *
Mito	0.22 ± 0.09	0.24 ± 0.10	0.22 ± 0.08	0.24 ± 0.15	0.24 ± 0.18	0.20 ± 0.11	0.18 ± 0.10	0.19 ± 0.11
Citrate								
Cyto	1.33 ± 0.15	1.35 ± 0.32	1.40 ± 0.35	1.35 ± 0.30	1.79 ± 0.70	0.96 ± 0.31	0.58 ± 0.07 *	0.53 ± 0.12 *
Mito	2.54 ± 1.10	2.90 ± 0.60	3.10 ± 1.26	3.23 ± 0.24	3.64 ± 0.62	2.24 ± 0.35	1.68 ± 0.31 *	1.38 ± 0.26 **
Pyruvate								
Cyto	0.12 ± 0.03	0.09 ± 0.05	0.10 ± 0.01	0.10 ± 0.08	n.d.	n.d.	n.d.	n.d.
Mito	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detectable.

Compartmentation of carboxylic acids related to the Krebs cycle (Table IV)

Under oxygenated conditions, the malate concentration slowly increased in both compartments. Cytosolic as well as mitochondrial concentrations of other carboxylic acids were stable. No concentration gradients between the subcellular spaces were found for malate and 2-oxoglutarate, whereas mitochondrial citrate exceeded the cytosolic value by a factor of 2. No pyruvate was detectable within the mitochondrial compartment.

Anoxia led to an immediate increase of cytosolic malate, the value for 30 min being 10-times higher than the corresponding value under oxygenated conditions. The increase of mitochondrial malate had a similar kinetic, but was of smaller magnitude, which led to a cytosolic/mitochondrial concentration gradient of about 2. The cytosolic 2-oxoglutarate concentration decreased to almost undetectable levels during anoxia, while mitochondrial 2-oxoglutarate was unaffected, leading to a mitochondrial/cytosolic concentration gradient of about 4 at the end of the experiment. Citrate concentrations decreased during oxygen deficiency, with the mitochondrial/cytosolic concentration gradient slightly increasing the 2.6 after 1 h. The cytosolic pyruvate concentration decreased to levels below the detection limit (0.01 mmol/liter).

Discussion

In the present study, the cytosolic and mitochondrial concentrations of key metabolites of energy metabolism and the malate–aspartate cycle were measured in order to estimate the concentration gradients of these compounds across the inner mitochondrial membrane within the intact cardiac cell. The data may contribute to a more profound understanding of the interactions between the cytosolic and the mitochondrial compartment and of the mechanism by which electrons are transported into the mitochondria.

Numerous studies have shown that myocardial oxygen deficiency, whether induced by hypoxia or ischemia, leads to conversion of glutamate, an intermediate of this cycle, to succinate and alanine [16–22]. In order to gain an insight into the mechanism of this metabolic rearrangement, the compartmentation of metabolites was also studied during anoxia.

For calculation of metabolite concentrations, the volumes of the subcellular water spaces had to be determined. Using ^3H_2O, the mitochondrial water space was estimated to be 35% of the total cellular water space, which agrees excellently with morphometric studies in the intact heart [23], whereas Geisbuhler et al. [9] reported a value of 26% using the same method as used here. Their absolute values of cytosolic and mitochondrial water spaces were also lower compared to the values obtained in this study.

Compartmentation of adenine nucleotides and phosphocreatine (Table II)

Of the cellular ATP, approx. 90% is present in the cytosolic space at a concentration of 8 mM. Values of 6, 10 and 11 mM have been measured in arrested rat hearts, working guinea-pig hearts and isolated myocytes, respectively [24,25,9]. The mitochondrial values were 2.8, 5.6 and 7.4 mM, respectively, in these studies compared to 2 mM in the present work. Studies of Soboll et al. [26] indicated that differences in the compartmentation of metabolites in isolated cells and whole tissue as determined by the digitonin method and the nonaqueous fractionation technique, respectively, are due to the model used and not to systematic errors inherent in one of these methods. Comparison of the two fractionation techniques using the same preparation, i.e., hepatocytes, yielded very similar results [27]. The discrepancies between the present study and the work of Geisbuhler et al. [9], who used exactly the same approach, are due mainly to the lower subcellular water spaces obtained there, leading to higher concentrations with the subcellular contents being approximately equal.

The contents of ADP in extracts of intact cells and cells treated with digitonin (4.2 and 1.8 nmol/mg protein, respectively) are comparable to the study of Geisbuhler et al. [9], but, as has been extensively discussed there, cytosolic and mitochondrial concentrations calculated from these data may not represent the true values due to release of protein bound ADP during fractionation. The cytosolic value of 0.37 to 1.07 mmol/liter overestimates the value calculated from the crea-

tine phosphokinase equilibrium (50 μmol/liter [28]) by a factor of 7–20.

Our data also corroborate the finding of Geisbuhler et al. [9], who demonstrated that AMP is mainly mitochondrial at a concentration of about 1 mM. Assuming a cytosolic concentration of 50 μM of ADP [28], an ATP concentration of 8 mM (Table I) and an equilibrium constant for adenylate kinase of $K_{eq} = 1.12$ [29], the free cytosolic AMP concentration may be as low as 0.4 μM, which is far below the detectable level.

Phosphocreatine should be confined to the cytosolic space. This can be deduced from the absence of creatine phosphokinase from the mitochondrial matrix [30], but was also measured in the intact heart of guinea-pigs and rats [25,31] and is corroborated by our results. On the other hand, Kauppinen et al. [24] found considerable amounts of this compound in the mitochondrial space of Langendorff-perfused rat hearts (20–50% of the cytosolic concentrations), the reason for this controversy remaining unclear.

During anoxia, cytosolic phosphocreatine progressively decreased (Table I), which should lead to a decrease of the ratio ATP/ADP_{free}, as these metabolites are connected by the equilibrium reaction catalyzed by creatine kinase. Indeed, cytosolic ATP slowly decreased, but even after 1 h of incubation, the ATP concentration was reduced by less than 20%. The maintenance of these high levels of high-energy phosphates is certainly due to the high rate of anaerobic glycolysis (Table I) and the low energy demand of the resting cells.

Compartmentation of intermediates of the malate–aspartate cycle

Studies on the malate–aspartate cycle, which is essential for the maintenance of oxidative metabolism in the heart, have been performed mainly on isolated mitochondria [3], while experiments using intact cells or tissues are scarce [32]. Mitochondrial and cytosolic isoforms of malate dehydrogenase, glutamate-oxaloacetate transaminase and membrane-carrier systems are involved in this cycle. Exchange of malate for 2-oxoglutarate is probably catalyzed by an electroneutral antiporter, while exchange of aspartate for glutamate is electrogenic by the co-transport of protons. By the latter mechanism, the protonmotive force generate by the respiratory chain can be used to channel reducing equivalents from the cytosol into the more reduced mitochondrial matrix during concerted action of the enzymes and carriers [1].

Under oxygenated conditions, stable concentrations of all the metabolites involved were maintained over a period of at least 30 min, except for malate, which slowly increased in the cytosol. Therefore, the values obtained for 30 min were assumed to be representative for the metabolic status of the cells. Cytosolic/mitochondrial gradients were found for aspartate ($c/m = 3$) and glutamate ($c/m = 2$), while malate and 2-oxoglutarate concentrations were similar in the two compartments.

These findings contradict results obtained by Kauppinen et al. [33,34], who measured compartmentation of metabolites in arrested rat hearts and found cytosolic/mitochondrial gradients of 2-oxoglutarate ($c/m = 0.1$), aspartate ($c/m = 32$) and glutamate ($c/m = 0.3$). However, these authors might overestimate mitochondrial values to some extent as judged from the compartmentation of phosphocreatine reported by this group [23], which might explain the low cytosolic/mitochondrial gradients of 2-oxoglutarate and glutamate in arrested hearts. Additionally, differences between experimental models exist (see above), which might explain the completely different results concerning aspartate.

Kauppinen et al. [33,34] used the cytosolic-mitochondrial distribution of anions to calculate the mitochondrial transmembrane pH gradient in situ, as it has been shown previously [35] that in suspensions of isolated mitochondria, anions are distributed according to the pH gradient. Using this approach, the lack of a transmembrane gradient of 2-oxoglutarate and malate in isolated, aerobic myocytes (Table IV) would indicate that there is no transmembrane pH gradient in these cells. However, comparison of the pH gradient measured using the probe dimethyloxazolidinedione [36] with the gradient calculated using the distribution of anions showed that the values obtained are not always in accordance [37]. This and the fact that the absence of a proton gradient in aerobic myocytes is very unlikely suggest that, within the intact cell, where anions are involved in several pathways, e.g., the malate–aspartate cycle

or the Krebs cycle, other concentration gradients may be maintained than those dictated by the proton gradient.

In addition, the anion-distribution approach assumes that the activity of the translocators is high enough to maintain near-equilibrium of the anions across the mitochondrial membrane. This assumption might not be valid for heart muscle, where the activity of the dicarboxylate–phosphate translocator, which connects the distribution of dicarboxylates to the proton gradient, is low [38]. Therefore, high transmembrane gradients are not to be expected in heart muscle, which is confirmed by our data. This is also true for citrate ($c/m = 0.5$), which is also transported by a carrier with low activity, the tricarbcxylate translocator [38].

Using the data for 2-oxoglutarate (Table IV), aspartate and glutamate (Table III), the cytosolic and mitochondrial concentrations of oxaloacetate, which is also involved in the malate–aspartate cycle, can be calculated to be 0.0026 and 0.0022 mmol/liter, respectively, assuming that the glutamate-oxaloacetate transaminase is in equilibrium in the two compartments ($K_{eq} = 6.6$ [39]). From these values and the concentrations of malate (Table IV), NADH/NAD ratios of $3.2 \cdot 10^{-3}$ for the cytosol and $5.7 \cdot 10^{-3}$ for the mitochondria can be calculated, using a K_{eq} of $2.8 \cdot 10^{-5}$ for malate dehydrogenase [39]. The mitochondrial value certainly is an underestimation, because in this compartment not all of the oxaloacetate may be available to the malate dehydrogenase because of the effective competition of citrate synthase for this substrate [4].

Using these data, we propose that the distribution of metabolites during normoxic, steady-state conditions supports the requirements of the malate-aspartate-cycle in the following way (Fig. 1). In order to achieve transport of electrons from the cytosol into the more reduced mitochondrial compartment, mitochondrial aspartate is extruded into the cytosol against a concentration gradient of $c/m = 3$ in exchange for glutamate, which follows a concentration gradient of only $c/m = 2$. The energy required is provided by the protonmotive force via the co-transport of a proton [3]. This exchange mechanism maintains a high ratio of glutamate/aspartate ($GLU/ASP_{mito} = 15$) in the mitochondrial compartment, which promotes

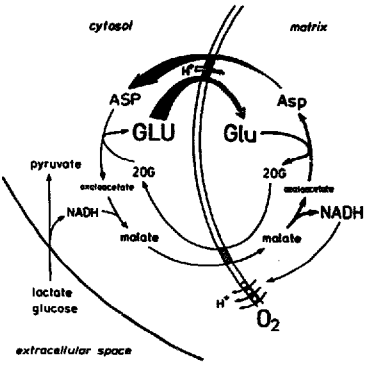

Fig. 1. Transport of reducing equivalents from the cytosol into the mitochondrial matrix by the malate–aspartate cycle. The process is facilitated by a cytosolic/mitochondrial concentration gradient for glutamate, the co-transport of a proton and a high mitochondrial glutamate/aspartate ratio. Transamination of oxaloacetate supports the generation of NADH in the mitochondrial matrix.

transamination of oxaloacetate. The withdrawal of oxaloacetate in turn supports the oxidation of malate, despite the unfavorable equilibrium condition of the malate dehydrogenase reaction and the highly reduced redox state, thus liberating the reducing equivalents that have been 'trapped' by the cytosolic malate dehydrogenase reaction.

In the cytosolic compartment, the lower GLU/ASP ratio ($GLU/ASP_{cyt} = 10$) facilitates the formation of oxaloacetate as an acceptor for NADH generated by glyceraldehyde-phosphate dehydrogenase or lactate dehydrogenase.

Effects of oxygen deficiency on the compartmentation of metabolites of the malate–aspartate cycle

Oxygen deficiency led to an instant rise of malate in both compartments (Table IV), and to a rapid stimulation of glutamate transamination with pyruvate in the cytosol, leading to the formation of alanine (Table III). At the same time, the cytosolic 2-oxoglutarate concentration decreased (Table IV) and succinate was released by the cells (Table I).

We suggest that the following events lead to these metabolic rearrangements (Fig. 2). Anoxia leads to an increase of the cytosolic NADH/NAD ratio, which was $1.3 \cdot 10^{-2}$ after 30 min – which is 4-fold higher than the value calculated for normoxic cells ($3.2 \cdot 10^{-3}$). The mitochondrial NADH/NAD ratio increased by a factor of 1.3, which is in accordance with results obtained in the hypoxic perfused rat heart using surface fluorometry [32]. Thus, transport of electrons from the cytosol into the mitochondria cannot keep in pace with glycolytic NADH production and may even be completely inhibited during anoxia. A crucial step causing the observed metabolic rearrangements may be the cessation of the energy dependent glutamate/aspartate exchange due to the collapse of the proton-motive force following anoxia. However, in a very recent study, Andersson et al. [40] demonstrated that the electrochemical potential is largely preserved during anoxia in isolated hepatocytes. Their results indicated that energy-dependent transport processes across the mitochondrial membrane are inhibited by other factors related to electron flow during anoxia. In any case, if glutamate–aspartate exchange ceases, trapping of mitochondrial oxaloacetate by transamination is impeded. Together with the increase of the mitochondrial NADH/NAD ratio, the oxidation of malate to oxaloacetate becomes extremely unfavorable and the malate concentration rises (Table IV).

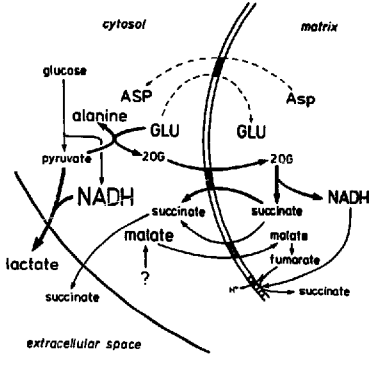

Fig. 2. Rearrangement of the malate–aspartate cycle during anoxia. The increase of intramitochondrial succinate following anoxia activates the exchange reaction with cytosolic 2-oxoglutarate. The decrease of cytosolic 2-oxoglutarate and the cessation of glutamate transport into the matrix stimulates transamination of glutamate with glycolytic pyruvate due to a mass action effect. Transfer of electrons generated in Krebs-cycle reactions to fumarate reductase may occur.

At the same time, succinate accumulate because oxidation of the flavoprotein-dependent succinate dehydrogenase can occur only by the respiratory chain and is strictly dependent on oxygen. Isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase may still be active, as can be deduced from the continuous decrease of the citrate pools (Table IV), which are not replenished due to impaired production of acetyl-CoA from pyruvate. The intramitochondrial succinate concentration may rise to values high enough to stimulate its outward transport by the anion carrier systems, which have a comparably low affinity for this substrate [3]. The counterion may be cytosolic 2-oxoglutarate, leading to a decrease of this compound in the cytosol. The depletion of cytosolic 2-oxoglutarate together with the inhibition of inward transport of glutamate might then stimulate cytosolic transamination of glutamate with pyruvate, yielding alanine, due merely to a mass action effect.

The result of the metabolic rearrangement outlined above is a conversion of the carbon skeleton of glutamate via 2-oxoglutarate to succinate within the mitochondria. Reoxidation of the NADH generated during this process might be achieved even under complete anoxia by the reduction of malate via fumarate to succinate. This reaction has been described in submitochondrial particles [41] and recently also in intact rat heart myocytes provided with appropriate precursors [42]. The origin of the malate needed to achieve redox balance remains an open question, as aspartate, the proposed precursor according to Taegtmeyer [17], is not available in sufficient amounts.

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