THE GLUTAMATE DEHYDROGENASE SYSTEM AND THE REDOX STATE OF MITOCHONDRIAL FREE NICOTINAMIDE ADENINE DINUCLEOTIDE IN MYOCARDIUM

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Received 21 April 1981

1. Introduction

Knowledge of the redox state of the free NAD^+/NADH couple in the mitochondria is essential in assessing the state of mitochondrial terminal oxidations. This is particularly important because the principal level of regulation of cellular respiration in intact tissues such as the heart is still in dispute. The myocardial mitochondrial free NAD^+/NADH ratio has, in a few instances, been estimated from the ratio of [2-oxoglutarate] $[NH_4^+]$/[glutamate] in the tissue, assuming that the glutamate dehydrogenase reaction is in equilibrium in myocardium [1–3]. The usefulness of the NAD-linked glutamate dehydrogenase reaction in heart muscle has not found general acceptance, and its systematic validation is lacking. In the liver, the β-hydroxyburyrate dehydrogenase and glutamate dehydrogenase reactions are apparently in near equilibrium [4], but in the heart the activity of the former enzyme is 1/2, and that of the latter 1/3 of that found in the liver [4]. For these reasons and because the metabolic capacity of the heart for ketone bodies is high and their intracellular concentrations are low, it was pointed out [2] that calculations of the mitochondrial free NAD^+/NADH ratios in heart tissue must be tempered with reservations.

These experiments on isolated perfused rat hearts and suspensions of isolated heart mitochondria indicate that the glutamate dehydrogenase reaction can be used as an indicator of the redox state of the mitochondrial free NAD^+ pool which is in equilibrium with the mitochondrial high fluorescence flavoprotein. Validation of this indicator metabolite method is especially important because the data obtained show that the mitochondrial respiratory chain in the heart operates at higher reduction levels of NAD^+ and a higher phosphorylation state of the adenine nucleotides [2,5] than liver mitochondria in situ [6].

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2. Material and methods

2.1. *Animals and perfusion method*

Female Sprague-Dawley rats from the Department's stocks were used. The rats were anaesthetized with diethyl ether and injected intravenously with 500 IU heparin before excision of the heart. Isolated hearts were perfused by the Langendorf procedure [7] without recirculation at a hydrostatic pressure of 7.85 kPa (80 cm H_2O) with Krebs-Ringer solution [8] equilibrated with O_2/CO_2 (19:1) and containing 2.5 mM Ca^{2+}. The experiments were preceded by a 10 min preperfusion with a substrate-free medium. The hearts were electrically paced to a frequency of 5 Hz and the pulse pressure, coronary flow, oxygen concentration in the venous effluent and organ surface flavoprotein fluorescence continuously monitored.

2.2. *Experiments on isolated mitochondria*

Rat heart mitochondria were isolated as in [9]. The experiments were conducted in 2.0 ml incubation mixture containing (final conc.) 10 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$, 10 mM potassium phosphate (pH 7.2), 210 mM sucrose and 1.6–2.2 mg mitochondrial protein/ml. The reactions were terminated by the addition of 0.2 ml 70% $HClO_4$.

2.3. *Metabolite assays*

Hearts were quick-frozen with aluminium clamps

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precooled in liquid nitrogen, pulverized in the frozen state and extracted with $HClO_4$ as in [3]. Metabolites in the neutralized $HClO_4$ extracts were assayed by enzymatic methods. Lactate was determined as in [10], pyruvate [11], 2-oxoglutarate [12], glutamate [13] and ammonia as in [14].

2.4. *Fluorometry and spectrometry of whole organs and mitochondrial suspensions*

Whole organ surface fluorometry was performed with a laboratory-built fluorometer [15], and for studies on isolated mitochondria an Aminco filter fluorometer was used. For the measurement of flavin fluorescence a Farrand 465 nm interference filter and a Corning 3494 filter were used as the primary and secondary filters, respectively. For the nicotinamide adenine nucleotide fluorescence the primary filter was a Corning 5840 and the secondary filter a combination of Corning 4303 and 3387. In the experiments depicted in fig.2 a custom-built apparatus for simultaneous measurements of the reflectance spectrum and fluorescence changes of perfused organs was used.

3. Results and discussion

3.1. *Activity of glutamate dehydrogenase*

The reported values of glutamate dehydrogenase in heart muscle homogenate are at variance [4,16,17] and they mostly refer to the favoured direction of the reaction, i.e., glutamate formation. We measured the ammonia formation in suspensions of intact mitochondria, which probably is the best indicator of in situ activity of the enzyme in the less-favoured direction of deamination. In the presence of 5 mM glutamate the rate of ammonia production was 5.1 nmol . min^{-1} . mg mitochondrial $protein^{-1}$ in state 3. Taking the myocardial content of mitochondria as 450 mg mitochondrial protein/g dry wt [18] the ammonia production was 2.30 μmol . min^{-1} . g myocardial dry wt^{-1} at 30°C. This value is close to the 2.47 μmol . min^{-1} . by dry wt^{-1} at pH 7.5 obtained in extracts of the heart [17]. The value is 15-times higher than the total ammonia production corrected for amide formation in an isolated perfused heart [17]. This high activity compared with the actual metabolic flux could be considered to result in a near equilibrium in the glutamate dehydrogenase reaction.

3.2. *Glutamate dehydrogenase as a redox indicator in myocardium*

In isolated heart mitochondria the glutamate dehydrogenase reaction reaches equilibrium quite rapidly, as depicted in fig.1. In uncoupled, rotenone- and arsenite-inhibited mitochondria the mitochondrial NAD^+-pool can be titrated with glutamate, 2-oxoglutarate and ammonia, and new steady states are attained within two minutes of changing the ratio of the components of the redox reaction.

In the isolated perfused heart the state of the glutamate dehydrogenase system was tested against the flavoprotein fluorescence, an indicator of the redox state of the mitochondrial free NAD^+ pool [19]. When the metabolic situation was changed by changing the substrate composition or oxygen content of the perfusion medium, the reactant concentrations of the glutamate dehydrogenase changed towards reduction concomitantly with quenching of the flavoprotein fluorescence (table 1, fig.2). Monitoring of the oxygenation state of myoglobin (fig.2) demonstrated that the fluorescence changes observed after substrate additions were not due to changes in the tissue oxygen

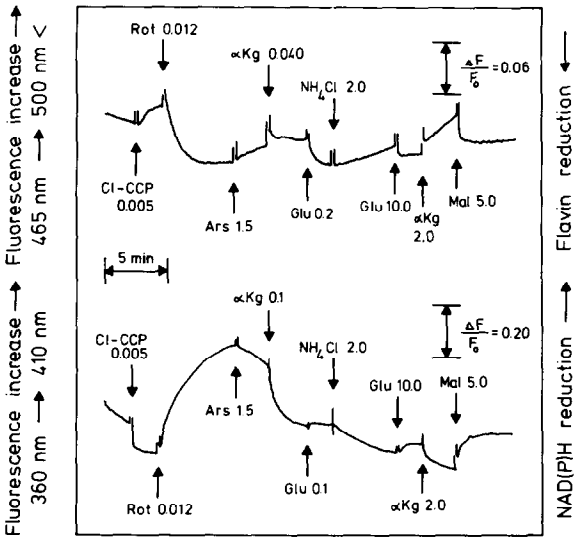


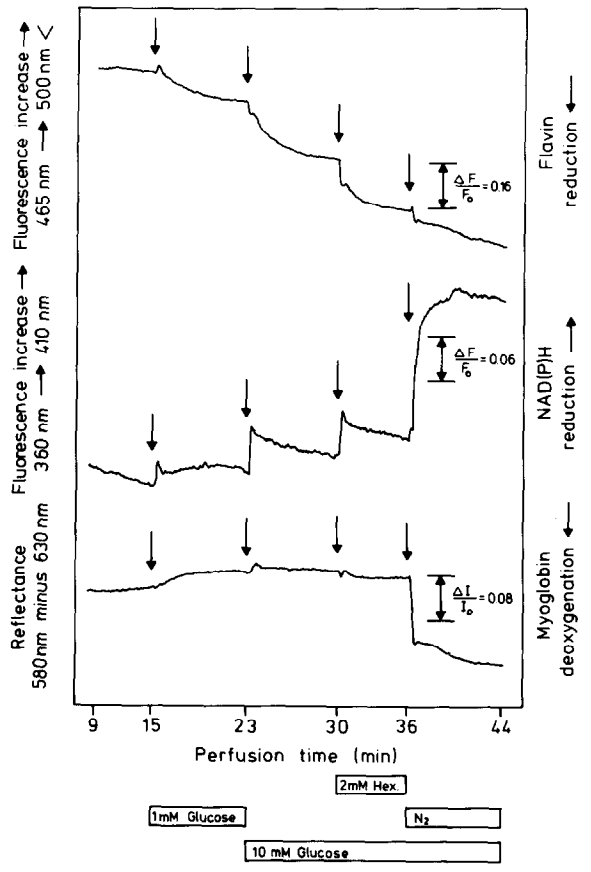
Fig.1. The glutamate dehydrogenase system and the redox states of fluorescent flavoproteins and the NAD(P)H/NAD(P) couple in isolated rat heart mitochondria. Experimental conditions were as section 2; total vol. 2 ml. *Abbreviations:* (Cl-CCP) carbonyl cyanide-*m*-chlorophenyl hydrogenase; (Rot) rotenone; (Ars) sodium arsenite; (α-Kg) 2-oxoglutarate; (Glu) glutamate; (Mal) Malate. The amounts added are expressed as μmol. To monitor the redox state of flavoproteins, the fluorescence above 500 nm was measured.

Table 1

Effect of perfusate substrate and oxygen concentrations on the oxidation-reduction equilibria of cytosolic and mitochondrial NAD^+-linked dehydrogenases

Perfusate compositions	Lactate	Pyruvate (μmol/g dry wt)	L/P	2-oxoglutarate	Glutamate (μmol/g dry wt)	NH_4^+	$\frac{NADH_m}{NAD^+_m}$
–	0.58 ±0.06	0.103 ±0.005	5.2	0.142 ±0.008	6.79 ±0.50	1.25 ±0.04	0.823 ±0.065
1 mM glucose	0.88 ±0.09	0.083 ±0.008	10.9	0.166 ±0.009	15.45 ±1.10	2.13 ±0.20	1.073 ±0.073
10 mM glucose	1.88 ±0.17	0.226 ±0.015	8.7	0.285 ±0.006	20.74 ±0.54	0.897 ±0.073	1.963 ±0.162
10 mM glucose + 2 mM hexanoate	2.75 ±0.043	0.064 ±0.004	41.8	0.169 ±0.010	28.66 ±0.53	0.958 ±0.064	4.102 ±0.212
10 mM glucose + N_2	18.14 ±3.38	0.083 ±0.004	219.1	0.029 ±0.001	14.50 ±1.06	1.19 ±0.19	11.76 ±2.90

Isolated rat hearts were perfused for 10 min with a medium containing no added oxidizable substrate, or containing 1 mM glucose, 10 mM glucose or 10 mM glucose + 2 mM hexanoate in medium equilibrated with O_2/CO_2 (19:1), or with 10 mM glucose in medium equilibrated with N_2/CO_2 (19:1). The mitochondrial free NADH/NAD^+ ratio ($NADH_m/NAD^+_m$) was taken as 3.87×10^{-3}. [glutamate]/[2-oxoglutarate][NH_4^+] and the ammonia content was converted to concentration by assuming an even distribution in the total tissue water. The mean dry wt/wet wt ratio was 0.13. Results are means ± SEM of 4–8 independent expt



concentration secondary to changes in oxygen consumption. Moreover, when the flavoprotein fluorescence changes were plotted against the changes in the glutamate dehydrogenase system in a double reciprocal manner [20], a straight-line was obtained (fig.3). Similar plots of the flavin fluorescence against the lactate/pyruvate ratio were not linear, in contrast to observations on rat liver [21], and neither was the plot of the tissue NADH fluorescence against the lactate/pyruvate ratio (not shown). This could indicate that the lactate dehydrogenase reaction is not at equilibrium with the total bound NADH/NAD^+ pool in heart muscle. This situation is different from that in the liver where an equilibrium between the total bound NADH/NAD^+ pool and extracellular lactate and pyruvate has been demonstrated [20]. Another line of evidence also points to the lack of equilibrium. The tissue NADH fluorescence did not respond to

Fig.2. The effect of oxidizable substrates on the redox states of fluorescent flavoproteins and nicotinamide nucleotides and oxygen saturation of myoglobin in isolated perfused rat heart. Surface fluorescence at wavelengths attributed to flavins and nicotinamide nucleotides and the epicardial reflectance difference at wavelengths of 580 and 630 nm were monitored as in section 2.

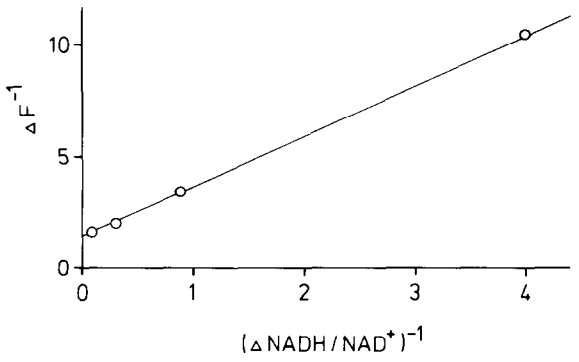


Fig.3. Double reciprocal plot of changes in the fluorescence of flavoproteins and changes in the mitochondrial NADH/NAD ratio, calculated from the tissue concentrations of the reactants in isolated perfused rat hearts, and the equilibrium constant of the glutamate dehydrogenase reaction.

The line was fitted by least-squares regression analysis, $r = 0.9999$.

changes in the lactate/pyruvate ratio of the perfusate upon changing the pyruvate concentration. The fluorescence changes merely suggested that the lactate dehydrogenase reaction did not readily operate in the direction of pyruvate reduction in the heart, which could be predicted from the kinetic properties of heart lactate dehydrogenase [22]. Titration of the high-fluorescence flavoprotein with β-hydroxybutyrate and acetoacetate in the perfusate revealed that flavin reduction of equal magnitude was caused by β-hydroxybutyrate or acetoacetate, and the flavin redox state did not respond to changes in the substrate ratio. Therefore, β-hydroxybutyrate dehydrogenase is not in equilibrium in cardiac muscle.

The substrate titrations presented in table 1, fig.2 and fig.3 indicate that the mid-potential of the fluorescent flavoprotein species is −314 mV. This value is somewhat lower than the E_m of −305 mV of the high fluorescence flavoprotein of rat liver mitochondria [19]. The value, however, is close enough to the E_m of −320 mV of the free NADH/NAD^+ couple to be a sensitive redox indicator of the mitochondrial free NAD pool.

In conclusion, in rat heart muscle the glutamate dehydrogenase reaction is in equilibrium with the high-fluorescence flavoprotein of the tissue. It is highly probable that this flavoprotein is the same as the high-fluorescence flavoprotein of the liver, i.e., lipoamide dehydrogenase [19,23] known to be in equilibrium with the mitochondrial free NADH/NAD^+ pool. This makes the reactants of the glutamate dehydrogenase useful redox indicators of intact myocardium. Moreover, lactate dehydrogenase, the reactants of which are widely employed as cytosolic redox indicators, may not catalyze an equilibrium reaction in isolated hearts perfused with external lactate and pyruvate.

Acknowledgements

This investigation was supported by grants from the Medical Research Council of the Academy of Finland and the Finnish Foundation for Cardiovascular Research. We are indebted to Miss Liisa Äijälä and Mrs Aila Holappa for their skillful technical assistance.

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