Biochimica et Biophysica Acta, 681 (1982) 300–304
Elsevier Biomedical Press

BBA 41130

ADENINE NUCLEOTIDE POOL SIZE, ADENINE NUCLEOTIDE TRANSLOCASE ACTIVITY, AND RESPIRATORY ACTIVITY IN NEWBORN RABBIT LIVER MITOCHONDRIA

JILL RULFS * and JUNE R. APRILLE **

Department of Biology, Tufts University, Medford, MA 02155 (U.S.A.)

(Received March 23rd, 1982)

Key words: Adenine nucleotide translocase; Adenine nucleotide; Respiration; Development; (Neonatal rabbit liver mitochondria)

The adenine nucleotide content (ATP + ADP + AMP) of newborn rabbit liver mitochondria was 6.0 ± 0.5 nmol/mg mitochondrial protein at birth, increased rapidly to 14.5 ± 1.7 nmol/mg protein by 2 h postnatal, peaked at 6 h, then decreased gradually to 7.8 ± 0.6 nmol/mg protein by 4 days postnatal. There was a strong positive correlation ($r = 0.82$) between the total adenine nucleotide pool size and adenine nucleotide translocase activity in these mitochondria. In contrast, glutamate + malate-supported State 3 respiratory rates remained constant from birth through the first week of life. State 4 rates also remained constant, as did the respiratory control index and uncoupled respiratory rates. The following conclusions are suggested: (1) The maximum rate of translocase activity is limited by the intramitochondrial adenine nucleotide pool size. (2) In newborn rabbit liver mitochondria, the State 3 respiratory rate is not limited by either the adenine pool size or the maximum capacity for translocase-mediated adenine exchange. (3) In contrast to rat, rabbit liver mitochondria are fully functional at birth with regard to respiratory rates and oxidative phosphorylation. (4) The rapid postnatal accumulation of adenine nucleotides by liver mitochondria, now documented in two species, may be a general characteristic of normal metabolic adjustment in neonatal mammals.

Introduction

In neonatal rat liver, the mitochondrial adenine nucleotide content (ATP + ADP + AMP) is very low at birth, but increases several-fold within 1–2 h of postnatal life [1–4]. The rapid increase in the matrix adenine nucleotide content may stimulate metabolic pathways which have an ATP- or ADP-dependent enzyme step localized to the mitochondrial compartment [4–6]. For example, gluconeogenesis is probably stimulated by the increase in pyruvate carboxylase activity that occurs when the matrix adenine pool is suddenly elevated [5]. Respiratory functions, including State 3 respiration and adenine nucleotide translocase activity, also increase postnatally as a function of the matrix adenine nucleotide content in neonatal rat liver [1–4]. Thus, the sudden accumulation of adenine nucleotides by liver mitochondria appears to have a central regulatory role in metabolic development during the first few hours of life. Furthermore, the rapid change in the respiratory functions which occur naturally after birth suggests a useful system for exploring fundamental questions on rate-limiting steps in oxidative phosphorylation [3].

The purpose of this study was two-fold. First, in view of the potential significance of this postnatal regulatory phenomenon, it was important to determine whether the rapid accumulation of

* Present address: Department of Biochemistry, Medical College of Virginia, Richmond, VA 23220, U.S.A.
** To whom correspondence and reprint requests should be addressed.

adenine nucleotides by liver mitochondria was a general characteristic of other mammalian neonates, e.g., the rabbit. Second, we used this model to investigate further the relationship between adenine nucleotide translocase activity and the State 3 respiratory rate during development.

Methods

Isolation of mitochondria. Full-term New Zealand White does were induced to deliver with an intramuscular injection of 1.25 USP units of oxytocin (Syntocinin). Pups were killed by decapitation at specific times, exsanguinated, and livers were removed to ice-cold isolation medium (0.225 M sucrose, 75 mM mannitol, 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4). The liver was minced, rinsed and homogenized in isolation medium (5 ml/g wet weight) using five strokes of a Contorque motor-driven Teflon/glass homogenizer. The homogenate was centrifuged for 10 min at $600 \times g$, at 4°C. The supernatant was then centrifuged at $8000 \times g$ for 10 min to pellet mitochondria. The pellet was washed three times by resuspending in isolation medium and centrifuging at $8000 \times g$ for 10 min. The final pellet was suspended to approx. 10 mg protein/ml in the same medium. Protein was determined by the method of Lowry et al. [7] using bovine serum albumin, fraction V as a standard.

Respiratory measurements. Respiratory measurements were made polarographically in a 1.0 ml water-jacketed glass chamber at 30°C using a Clark oxygen electrode (Yellow Springs Instruments). The assay medium consisted of 0.225 M sucrose, 1 mM EDTA, 5 mM $MgCl_2$, 15 mM KCl, 15 mM KH_2PO_4/K_2HPO_4, 50 mM Tris-HCl, pH 7.4. Mitochondria were added at a concentration of 0.5–1.0 mg protein/ml. Glutamate and malate were added to a final concentration of 5 mM each and State 3 respiratory activity was initiated by the addition of 150 nmol ADP. Rates were calculated as nmol $\frac{1}{2}O_2$/min per mg protein. Respiratory control indices were determined as the numerical ratio of the State 3 to the State 4 rate. Uncoupled respiratory rates were determined in the presence of 40 μM 2,4-dinitrophenol.

Extraction and assay of adenine nucleotides. Mitochondria were extracted for the determination of adenine nucleotides by mixing equal volumes of mitochondrial suspension (5–10 mg protein/ml in the isolation medium) and 12% $HClO_4$. After centrifuging, the supernatant was neutralized to pH 6.8–7.2 with 1.65 M K_2CO_3, 0.43 M triethanolamine and centrifuged to clear $KClO_4$. The extract was assayed for adenine nucleotides by enzymatic methods [7,8].

Adenine nucleotide translocase activity. Translocase activity was measured at ice temperature using a forward exchange reaction [10]. Mitochondria were suspended to 1 mg/ml in isolation medium to a final volume of 1.0 ml. The assay was initiated by the addition of 20 μM [^{14}C]ADP ($3.5 \cdot 10^3$ dpm/nmol) and terminated at 10, 20, 30 and 60 s by rapid filtration of 0.3-ml aliquots through Millipore AAWP filters. Filters were washed once with 10 ml cold 0.15 M NaCl; radioactivity was determined by liquid scintillation counting. Nonspecific binding of isotope to filters and to mitochondria was estimated using a blank containing 100 μM atractyloside.

Results

Mitochondrial adenine nucleotide content

Total mitochondrial ATP + ADP + AMP increased 2–3-fold immediately after birth, from 6.0 ± 0.5 nmol/mg mitochondrial protein to 14.5 ± 1.7 nmol/mg at 2 h postnatal. Within 6 h after birth, the total adenine nucleotide pool size reached a peak, and then gradually decreased to

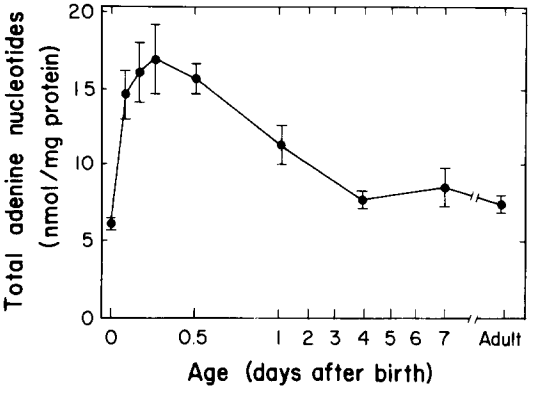

Fig. 1. Total adenine nucleotide content in isolated rabbit liver mitochondria during postnatal development. Each point is the mean ±S.E. for five to eight separate experiments.

TABLE I

ADENINE NUCLEOTIDE CONTENT IN ISOLATED RABBIT LIVER MITOCHONDRIA DURING POSTNATAL DEVELOPMENT

All values are the mean ±S.E. for *n* experiments. Units are nmol adenine nucleotide/mg mitochondrial protein.

Age	*n*	ATP+ADP	AMP	Total
Birth	12	4.1±0.4	2.1±0.3	6.0±0.5
4–6 h	4	13.9±1.3	2.2±0.3	16.2±1.5
1 day	7	8.9±0.7	2.4±0.3	11.3±1.3
4 day	8	6.0±0.2	1.8±0.2	7.8±0.6
7 day	4	6.9±0.4	1.9±0.5	8.7±1.1

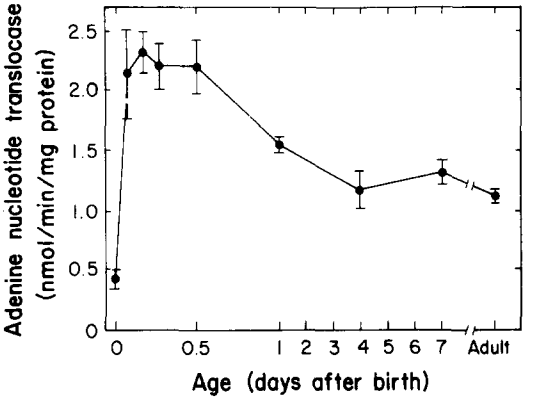

Fig. 2. Adenine nucleotide translocase activity in isolated rabbit liver mitochondria during postnatal development. Each point is the mean ±S.E. for four to seven separate experiments.

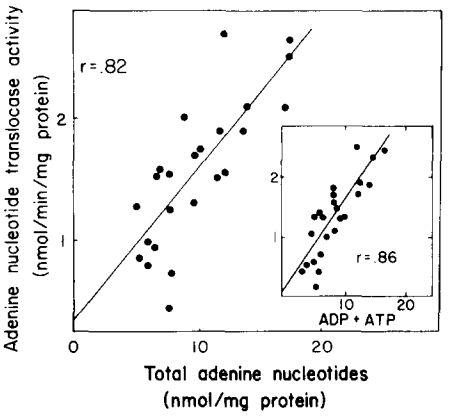

Fig. 3. Adenine nucleotide translocase activity as a function of total (ATP+ADP+AMP) or exchangeable (ATP+ADP; inset) adenine nucleotide content in isolated mitochondria. These data were obtained from the experiments represented in Figs. 1 and 2. The lines were determined by linear regression analysis; *r*, correlation coefficient.

adult levels by day 4 (Fig. 1). Changes in total adenine nucleotides were due to changes in ADP + ATP; the AMP pool size did not vary (Table I).

Adenine nucleotide translocase activity

Translocase activity also showed a sharp increase in the first 2 h after birth, followed by a gradual decrease to adult values by day 4 (Fig. 2). These changes directly paralleled those seen for the matrix adenine pool size (see Fig. 1). When translocase activity was examined as a function of total mitochondrial adenine nucleotide content (Fig. 3) or of the ATP + ADP pool (Fig. 3, inset), there was a strong positive correlation ($r = 0.82$ and 0.86, respectively).

TABLE II

RESPIRATORY ACTIVITY IN ISOLATED RABBIT LIVER MITOCHONDRIA DURING POSTNATAL DEVELOPMENT

Respiratory rates were measured polarographically with glutamate+malate as the substrate, and are in units of nmol 1/2 O_2/min per mg mitochondrial protein. RCI, respiratory control index (State 3/State 4). Uncoupled rates were determined with 40 μM 2,4-dinitrophenol. Each value is the mean ±S.E. for *n* experiments.

Age	*n*	State 3	State 4	RCI	Uncoupled
Birth	6	92.9± 7.2	16.0±5.0	5.9±0.6	120.0±11.8
4–6 h	4	95.7± 4.2	15.0±1.4	6.3±0.6	141.2±11.5
1 day	7	97.5±11.7	20.7±3.1	5.2±0.3	114.0± 5.6
4 day	8	98.6± 9.1	17.3±2.7	5.6±0.2	123.9±10.3
7 day	6	105.5±10.2	20.1±5.3	5.5±0.7	146.6± 9.2

State 3 respiration

With glutamate plus malate as substrate, State 3 respiratory rates showed no significant variation during the first 7 days of life (Table II). State 4 rates also remained constant, so that the respiratory control index was as high at birth as it was any time thereafter. Uncoupled respiratory rates were also invariant.

Discussion

The rapid increase in the mitochondrial adenine nucleotide content that occurred in rabbit liver within a few hours of birth was even more dramatic than that seen in rat [1–4]. In rat liver, the matrix adenine content plateaus at about 2 h and remains constant [3]. In the rabbit, a large initial overshoot occurred within 2–6 h, followed by a gradual return to adult levels over a period of several days. The close correlation between the adenine nucleotide content and adenine translocase activity during these fluctuations was convincing evidence that the rate of translocase-mediated adenine exchange was directly dependent on the size of the exchangeable adenine nucleotide pool in the matrix. This conclusion has been reported previously for isolated adult rat liver mitochondria, based on experiments in which conditions were manipulated to change either the proportions of exchangeable and nonexchangeable adenine nucleotides [11,12], or the total pool size [13] in the matrix. The data presented here, and in previous work with the newborn rat [3,4], confirm this important result, but in a natural in vivo model in which no experimental intervention was necessary, and in which the amount of AMP in the matrix remained constant. This system may prove useful for further fundamental investigation into the mechanism of adenine exchange over the carrier.

Despite wide fluctuations in the rate of translocase activity in the postnatal period, the State 3 respiratory rate remained invariant. This suggested that the coupled respiration rate was not limited by the rate of adenine nucleotide exchange. Translocase activity was measured here under nonphosphorylating conditions and at ice temperature, whereas State 3 respiration was at 30°C, so rates cannot be compared numerically. Nevertheless, a fractional increase in translocase activity should have resulted in a corresponding fractional increase in the State 3 rate if adenine exchange were rate limiting. Therefore, the rapid 4-fold increase in translocase activity after birth was noteworthy in that it was not accompanied by a change in the maximum rate of coupled respiration. This is different from the results reported for adult rat liver mitochondria by Lemasters and Sowers [14]. In their study, the rate of translocase activity was varied by titrating with the specific inhibitor, atractyloside; fractional changes in the rate of exchange were accompanied by similar fractional changes in State 3 rates. It is possible that this reflects a real difference between neonatal and adult liver mitochondria. However, we should note also that the inhibitor will block entirely the activity of individual carrier moieties whereas variations in the adenine pool size may affect more truly the rate of individual carriers. If carriers are spatially linked with the ATPase, as has been proposed [15], then the binding of the inhibitor atractyloside would be expected to affect the rate of oxidative phosphorylation more directly than would be supposed otherwise.

In our experiments, the external ADP concentration was saturating for the carrier, and no ATP was added. Therefore, it is important to distinguish that only the maximum capacity of the carrier was shown not to be rate limiting. If ADP and ATP are both present, the respiratory rate can be shown to vary as a function of the ATP/ADP ratio [16–18]. Under those conditions, the specificity of the carrier determines the rate at which ADP will enter the matrix. It is this specificity which may make the carrier rate limiting for respiration under physiological conditions [16–19], although this point has been debated [15,20,21].

Rabbit liver mitochondria were fully functional at birth with regard to oxidative phosphorylation. In contrast, neonatal rat liver mitochondria have low State 3 respiratory rates; however, the adenine nucleotide content is also much lower in the rat than in the rabbit [1–4]. In the rat, State 3 develops quickly, attaining a maximum within 30 min when the matrix adenine pool size is only slightly increased, from approx. 3 to 4–5 nmol/mg protein. Thus, a threshold of only 4–5 nmol/mg protein seems to be required for maximum rates of State 3 respiration [3,4]. This is consistent with the

observation that newborn rabbit liver mitochondria, which had over 6 nmol adenines/mg protein at the outset, also had fully developed rates of coupled respiration at birth. The State 4 rates were quite low, resulting in a respectable respiratory control index of about 6. The functional integrity of these isolated mitochondria appeared to be as good at birth as it was at any later time up to 7 days of age.

The rapid accumulation of adenine nucleotides from the liver cytosol into the mitochondria has now been documented in two species, the rat and rabbit, suggesting that this may be a general occurrence in mammalian neonates. In addition to regulating the development of coupled respiration (in the rat) and adenine translocase activity (rat and rabbit), the sudden increase in the ATP and ADP content may provide a mechanism for the rapid postnatal stimulation of other adenine nucleotide-dependent reactions in the matrix. There is evidence that neonatal gluconeogenesis may be regulated in this way via an increase in the ATP concentration available to the matrix enzyme pyruvate carboxylase [5]. Any ATP- or ADP-dependent matrix reaction for which the enzyme(s) are already present at birth is a possible candidate for such regulation.

In order for the uptake of adenine nucleotides to occur at all, a novel transport mechanism must be envisioned, since 1:1 exchange over the translocase could not account for net movements. This new transport system has been the subject of several recent reports [4,22–24].

Acknowledgements

This work was submitted by J.R. as part of a thesis for the Ph.D. in Biology at Tufts University. This work was supported by NIH Grant Nos. NS 14936 and HD 11697.

References

1 Nakazawa, T., Asami, K., Suzuki, H. and Yukawa, O. (1973) J. Biochem. (Tokyo) 73, 397–406
2 Sutton, R. and Pollak, J.K. (1978) Differentiation 12, 15–21
3 Aprille, J.R. and Asimakis, G.K. (1980) Arch. Biochem. Biophys. 201, 564–573
4 Aprille, J.R. (1981) Arch. Biochem. Biophys. 207, 157–164
5 Aprille, J.R., Yaswen, P. and Rulfs, J. (1981) Biochim. Biophys. Acta 675, 143–147
6 Goldstein, F.G. and Aprille, J.R. (1982) Arch. Biochem. Biophys. 213, 7–13
7 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
8 Lamprecht, W. and Trautschold, I. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 4, pp. 2101–2110, Verlag Chemie, Weinheim
9 Jaworek, D., Gruber, W. and Bergmeyer, H.U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 4, pp. 2127–2131, Verlag Chemie, Weinheim
10 Winkler, H.H., Bygrave, F.L. and Lehninger, A.L. (1980) J. Biol. Chem. 243, 20–28
11 Barbour, R.L. and Chan, S.P. (1981) J. Biol. Chem. 256, 1940–1948
12 Pfaff, E., Heldt, H.W. and Klingenberg, M. (1969) Eur. J. Biochem. 10, 484–493
13 Asimakis, G.K. and Aprille, J.R. (1980) Arch. Biochem. Biophys. 203, 307–316
14 Lemasters, J.J. and Sowers, A.E. (1978) J. Biol. Chem. 254, 1248–1251
15 Vignais, P.V. and Lauquin, G.J.M. (1979) Trends Biochem. Sci. 4, 90–92
16 Davis, E.J. and Lumeng, L. (1975) J. Biol. Chem. 250, 2275–2282
17 Davis, E.J. and Davis-van Thienen, W.I.A. (1978) Biochem. Biophys. Res. Commun. 83, 1260–1266
18 Küster, U., Bohnensack, R. and Kunz, W. (1976) Biochim. Biophys. Acta 440, 391–402
19 Küster, U., Letko, G., Kunz, W., Duszynski, J., Bogacka, K. and Wojtczak, L. (1981) Biochim. Biophys. Acta 636, 32–38
20 Holian, A., Owen, C.S. and Wilson, D.F. (1977) Arch. Biochem. Biophys. 181, 164–171
21 Owen, C.S. and Wilson, D.F. (1974) Arch. Biochem. Biophys. 161, 581–591
22 Sutton, R. and Pollak, J.K. (1980) Biochem. J. 192, 75–83
23 Asimakis, G.K. and Aprille, J.R. (1980) FEBS Lett. 117, 156–160
24 Aprille, J.R. and Austin, J. (1981) Arch. Biochem. Biophys. 212, 689–699