*Biochimica et Biophysica Acta* 851 (1986) 249–256
Elsevier



BBA 42103

# Compartmentation of high-energy phosphates in resting and beating heart cells

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(Received March 20th, 1986)

Key words: Creatine kinase; Metabolite compartmentation; High-energy phosphate; (Frog heart cell)

**The subcellular distribution of ATP, ADP, creatine phosphate and creatine has been analyzed by fast detergent fractionation of isolated frog heart cells. Digitonin fractionation (0.5 mg/ml, 10 s at 2°C in 20 mM 4-morpholinepropanesulfonic acid/3 mM EDTA/230 mM mannitol medium) was used to separate mitochondria and myofilaments from cytosol. To separate myofilaments from the other cellular compartments, Triton X-100 was used (2%, 15 s in the same medium as digitonin). For either resting or beating cells the total cellular contents of ATP, ADP, creatine phosphate and creatine was similar, nevertheless the $O_2$ consumption was 6-times higher. The compartmentation of these metabolites was also identical. Myofilaments contain 1.1 nmol ADP per mg total cellular proteins. In the cytosolic compartment the metabolite concentrations, all measured in nmol per mg total cellular proteins, were: ATP, 13; ADP, 0.25–0.05; creatine phosphate, 18.5 and creatine, 14. This indicated that the reaction catalyzed by creatine kinase was in a state of (or near) equilibrium.**

## Introduction

In a heart where an intense ATP consumption is necessary to maintain constant beating, the high-energy compound creatine phosphate is also present in high concentrations. Furthermore, this metabolite is susceptible to greater variations in cellular concentration than is ATP. The only reaction producing creatine phosphate is the so-called Lohman reaction catalyzed by creatine kinase:

$$\text{creatine} + \text{MgATP} \rightleftharpoons \text{creatine-phosphate} + \text{MgADP} + \text{H}^+$$

The equilibrium of this reaction does not favor creatine phosphate synthesis and it was formerly thought that creatine phosphate was a reservoir for energy and only used to maintain a high level of ATP concentration. According to this hypothesis, the creatine-kinase reaction is always in rapid equilibrium with the ATP-ADP couple. Observations obtained during the last 10 years have lead to another model called 'creatine shuttle' and described in the recent reviews by Bessman [1] and Jacobus [2]. In this hypothesis, creatine phosphate is the carrier of energy between the sites of high-energy phosphates synthesis, mitochondria and glycolysis and sites of ultilisation, myofilaments or membrane ATPases. The hypothesis of the 'creatine shuttle' implies that several forms of creatine kinase exist in the vicinity of the sites of synthesis or degradation of creatine phosphate, and that the Lohman reaction is not at equilibrium everywhere in the cell. In the most restrictive model of the shuttle recently described [1], "adenine nucleotides are considered to be compartmented at strategic sites with a high concentration of ATP only in the intramitochondrial space and of ADP at the

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Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

peripheral ATPase". The first condition of the model, localization of creatine kinase within cells, is well documented. The soluble isoforms MM, MB or BB are localized in the cytosol and MM is partially bound to myofilaments [3,4]. The mitochondrial form of creatine kinase is bound to the external face of the internal membrane of mitochondria [3].

The equilibrium of the reaction catalysed by creatine kinase or the existence of compartments where this reaction is not at equilibrium is a controversial topic. Although direct evidence is lacking, it is generally thought that the creatine kinase reaction is at equilibrium in the cytosolic compartment [5,6]. To obtain a direct invalidation or confirmation of the postulated equilibrium of the reaction catalysed by creatine kinase, the concentration of the enzyme's free substrates in the main cellular compartments must be determined. Several techniques have been described to separate cellular compartments (Soboll et al. [7]; Zuurendonk et al. [8]), but these techniques are only possible to use on cells in which two main compartments are encountered: mitochondrial and extramitochondrial. In the case of muscle cells, a third compartment is present: myofilaments. In this compartment it is known that ADP is bound to actin [9] and ADP or ATP is probably bound to myosin [10,11].

To separate the three main compartments in cardiac cells, mitochondria, myofilaments and cytosol, we have used the fast digitonin fractionation method [8] and developed a fast Triton X-100 fractionation. We utilized cells isolated from frog heart because in these cells the sarcoplasmic reticulum is not very extensive; furthermore, many electro-physiological and metabolic studies have been performed on frog. The cellular fractionation permitted the study of the high-energy phosphates in the main cellular compartments.

## Material and Methods

*Cell isolation*

Myocytes were isolated from frog heart (Rana esculenta) by enzymatic perfusion with collagenase and trypsin. The method was an adaptation of those described for the isolation of adult cardiac cells from rat or guinea pig [12]. Details of the isolation technique have been published elsewhere [13]. After isolation in a medium nominally $Ca^{2+}$ free, cells were preincubated in Ringer medium without calcium (88.4 mM NaCl/2.5 mM KCl/23.8 mM $NaHCO_3$/0.6 mM $NaH_2PO_4$/3.8 mM $MgCl_2$, bubbled with 95% $O_2$/5% $CO_2$) in the presence of 5 mM pyruvate as substrate and 0.1% fatty acid-free bovine serum albumin. After this preincubation step, 75–90% of the isolated cells were morphologically similar to frog heart cells in situ, i.e., spindle-like (200–300 $\mu$m, 5 $\mu$m diameter) and were resistant to physiological concentrations of calcium [13].

Cell incubations were usually performed at 22°C, in Ringer medium 0.9 mM $CaCl_2$, in the presence of 5 mM pyruvate as substrate and fatty acid-free bovine serum albumin 0.1%. When the activity of dehydrogenases other than lactate dehydrogenase was studied, the cells were incubated in 5 mM glucose instead of pyruvate. In this medium, the isolated cells are quiescent. When 5–10 mM KCl is added, the cells beat and a higher KCl concentration (60–80 mM) induces a contracture. The exact KCl concentration necessary to obtain beating was difficult to determine because it depended upon the resting potential of the isolated cells. Thus, we induced beating by adding barium ions [14] (0.5 mM $BaCl_2$). In both conditions the frequency of beating was 15–20 per min (0.25–0.33 Hz).

*Fast digitonin or Triton fractionation*

Fast fractionation was performed according to Zuurendonk et al. [8]. 1.5 ml Eppendorf centrifuge tubes were filled with; 0.2 ml 10% perchloric acid at the bottom, 0.5 ml silicon oil, 0.5 ml fractionation medium and maintained at 0°C during 1 h before use. A 0.15 ml aliquot of the cell suspension was added and mixed for 10 s at low temperature with the fractionation layer and then centrifuged for 40 s at maximum rate in either a TH 12 Janetzki centrifuge or in a MK2 refrigerated Sigma centrifuge ($9000 \times g$). 0.2 ml of the top layer was then removed and added to 2.5 $\mu$l 70% perchloric acid, 0.2 ml of the same layer was rapidly frozen to perform enzymatic tests. The two perchloric acid fractions (top and bottom) were neutralized as soon as possible with 5 M KOH containing 0.3 M Mops (from Merck). After removing oil and

soluble fraction the pellet was dissolved in 150 μl 1 M NaOH for protein determination.

The fractionation medium contained 20 mM morpholinopropanesulfonic acid, 3 mM EDTA, plus either 0.23 M mannitol or 0.115 M KCl and digitonin (usually 0.65 mg/ml) or 2% Triton X 100. Cell suspensions were also diluted and centrifuged under the same conditions, by using 0.5 ml Ringer medium instead of fractionation medium.

As the density of the cells is 1.06 g/ml [18] a silicon oil of density 1.03 g/ml was used, either 508V70 from Rhone Poulenc or a mixture of 2 volumes DC200 plus 10 volumes AR200 from Serva. For enzymatic activity determinations in the bottom fraction, perchloric acid was replaced by a Ringer medium of density slightly lower than the cell density.

*Metabolite and enzyme determination*

ATP, ADP and creatine phosphate determinations were performed by bioluminescence, using either an apparatus built by B. Arrio et al. [15] or a LKB 1250 luminometer. Luciferase was purified by electrofocusing from dried tails of fireflies (Sigma), according to Lundin et al. [26]. The ADP used for the creatine phosphate determination was purified by ion exchange chromatography on Dowex AG1X8.

Creatine was determined by fluorimetry [17]; with this method it was not possible to estimate amounts lower than 0.5 nmol; therefore, creatine was only determined on whole cell extracts.

Lactate dehydrogenase activity was determined as in Ref. 18, in a medium containing 50 mM potassium phosphate buffer (pH 7.4)/0.5 mM pyruvate/0.14 mM NaDH; changes in absorbance at 340 nm were recorded after addition of NADH. Glutamate dehydrogenase activity was assayed in a medium containing 50 mM potassium phosphate buffer (pH 7.4)/1 mM EDTA/2 mM ADP/0.04 M $(NH_4)_2SO_4$/0.14 mM NADH/4 mM NaCN/10 mM 2-oxoglutarate; changes in absorbance at 340 nm were recorded after addition of 2-oxoglutarate; NaCN was added to inhibit NADH oxydase activity. ATPases activities were studied at 30°C by a coupled enzymatic assay, in a medium containing: 50 mM triethanolamine buffer (pH 7.1)/75 mM KCl/0.1 mM EGTA/10 mM $MgCl_2$/1 mM ATP/27 μM phospho*enol*-pyruvate/15 μM NADH/2.0 IU/ml lactate dehydrogenase/1 IU/ml pyruvate kinase; the decrease of NADH fluorescence (Ex 340 nm, Em 460 nm) was recorded in a JY3D fluorometer after addition of the sample. The calcium sensitivity was tested by addition of $CaCl_2$ final concentration 0.2 mM and the possible inhibition by either 10 μM ouabain or 80 μM $NaN_3$.

Protein content was determined by the Lowry method [19]. Oxygen consumption was determined polarographically (Yellow Spring Instruments).

*Statistical evaluation*

Data were expressed ±S.D. and were statistically analyzed using the Student's *t* test.

## Results

*Properties of the isolated cells*

The protein content, determined on the pellet of whole cells centrifuged through silicon oil, averaged 0.4–0.6 ng per cell. Round cells were not eliminated by the centrifugation; therefore the amount of protein determined on the pellet corresponded to the spindle cells plus round cells.

Oxygen consumption was 10–11 natom/min per mg cellular protein for resting cells, and 60–66 for beating cells in the presence of either 10 mM KCl or 0.5 mM $BaCl_2$ (five cell isolations). If it is assumed that 1 mg total protein corresponds to 10 mg wet weight, these values correspond to those observed for the whole heart [20,21] or for isolated cardiocytes from rat [22–24].

As previously observed [13], no significant differences were observed between the total ATP, ADP, creatine and creatine phosphate levels in beating or resting cells. The ATP/ADP ratio was $6.1 \pm 1.5$ (S.D.) for quiescent cells and $5.1 \pm 0.9$ for beating ones, and the ratios of creatine phosphate/creatine were $1.4 \pm 0.3$ and $1.8 \pm 0.7$, respectively. These differences were not significant ($p \geqslant 0.05$) and the ratios were very similar to the observations on whole frog heart [25–27].

*Digitonin fractionation*

To determine the digitonin concentration at which the sarcolemma is lysed without lysis of the external membrane of mitochondria, cells were

mixed for 10 s at 4°C in fractionation medium containing various digitonin concentrations. After centrifugation in silicon oil, the supernatants were tested for the presence of marker enzymes, lactate dehydrogenase for cytosol and glutamate dehydrogenase for mitochondrial matrix [28]. As shown in Fig. 1, for a concentration 0.5 mg/ml of digitonin (i.e., 0.65 mg/ml before adding cell suspension in the Eppendorf centrifuge tube) 90% of the maximum cellular lactate dehydrogenase activity was released. The 10–20% lactate dehydrogenase found in the absence of digitonin corresponds to the 10–20% debris and round cells. A concentration of 1.5 mg/ml of digitonin was necessary to obtain the total lactate dehydrogenase in the supernatant. At a digitonin concentration of 0.5 mg/ml, the lysis of the sarcolemma was sufficient to release all the small metabolites of the cytosol into the medium, whereas the mitochondrial membrane was not damaged. For digitonin concentrations up to 5 mg/ml, no glutamate dehydrogenase was observed in the supernatant (Fig. 2A, curves C and D). When Percoll density 1.05 g/ml instead of perchloric acid was layered in the bottom of the centrifuge tubes, it was possible to observe

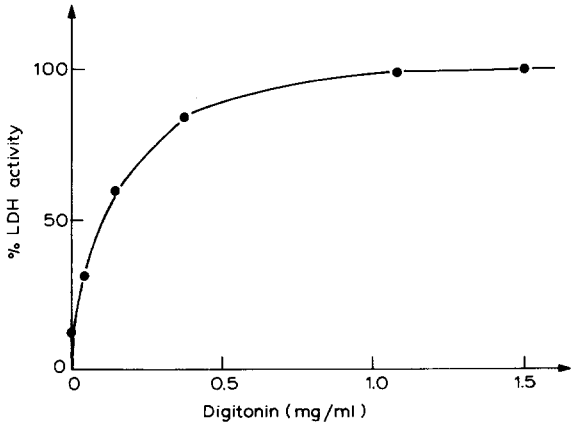


Fig. 1. Lactate dehydrogenase activity release of cell suspensions incubated for 10 s at 4°C in fractionation media containing varying concentrations of digitonin. 0.15 ml of cell suspension at 22°C were mixed with 0.5 ml fractionation medium at 0°C; the enzyme activity was determined in the supernate after centrifugation through silicon oil. The 100% activity was determined on cells treated with Lubrol.

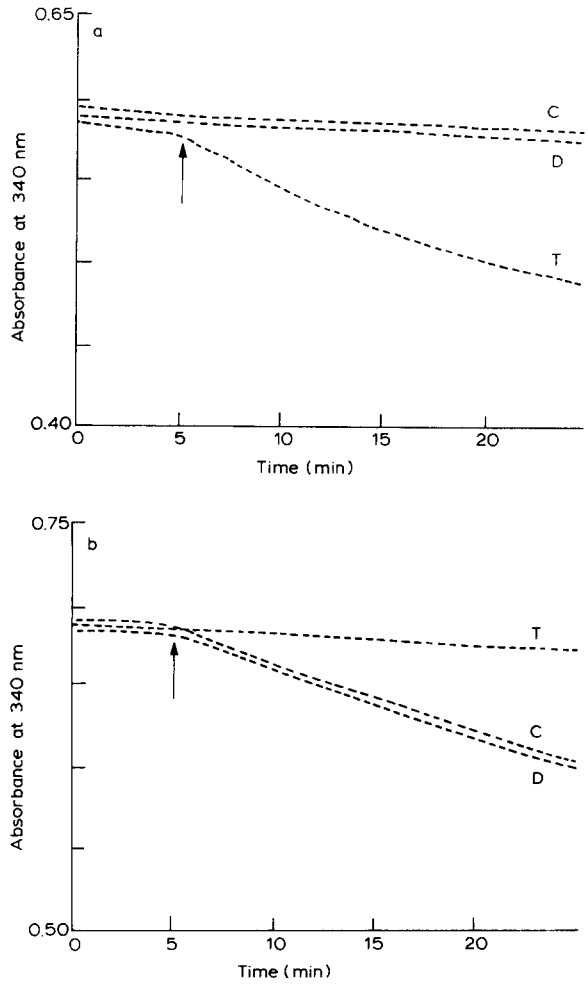


Fig. 2. Glutamate dehydrogenase activity in supernatant (Fig. 2A) or bottom (Fig. 2B) fractions obtained for: Control cells in Ringer medium (C); Cells lysed 10 s in 0.5 mg/ml digitonin at 4°C (D); Cells lysed 15 s in 2% Triton X-100 at 4°C (T). The decrease of NADH absorbance was studied in phosphate buffer (pH 7.4)/1 mM EDTA/2 mM ADP/0.04 M $(NH_4)_2SO_4$/4 mM NaCN; 10 mM 2-oxoglutarate was added after 5 min incubation.

glutamate dehydrogenase activity in the Percoll fraction (Fig. 2B, curves C and D).

*Triton X-100 fractionation*

Digitonin was effective for lysing the sarcolemma, but in the lysis medium in the presence of EDTA the myofilaments are maintained, as has been shown on rat heart cells [29,30]. In the lower layer after silicon oil centrifugation, an ATPase

activity was observed: this activity was insensitive to ouabain, but was partially inhibited by $NaN_3$ and activated by calcium: this indicates that both mitochondrial ATPase and actomyosin ATPase are present in the digitonin residue. Another medium was used to obtain solubilization of all parts of the cells except myofilaments. Triton X-100 is known to maintain the structure of myofilaments [31].

A technique equivalent to the fast digitonin fractionation was used, but the fractionation medium contained 2% Triton X-100 instead of digitonin and the cell suspension was mixed for 15 s at 4°C before centrifugation through silicon oil. The time necessary to obtain lysis was determined by the appearance of lactate dehydrogenase and glutamate dehydrogenase in the supernate (Fig. 2 curves T). After Triton X-100 fractionation, the bottom fraction contained an ATPase activated by calcium but insensitive to $NaN_3$ or ouabain: myosin ATPase.

The contents of the supernatants and bottom fractions obtained after centrifugation through silicon oil of the cell suspension in either Ringer medium ($S_R$ and $B_R$) or digitonin ($S_D$ and $B_D$) or Triton X-100 ($S_T$ and $B_T$) are summarized in the Table I.

TABLE I

CONTENT OF FRACTIONS OBTAINED AFTER FAST LYSIS OF FROG HEART CELLS BY EITHER DIGITONIN OR TRITON X-100

0.15 ml of cell suspension incubated at 22°C was quickly mixed with 0.5 ml of medium at 0°C; this medium (see Material and Methods) contained either 0.65 mg/ml digitonin or 2% Triton X-100 in Mops, EDTA buffer (pH 7.0), or Ringer medium as control. After centrifugation through silicon oil, the subcellular contents of the top (S) and bottom (B) fractions were deduced from the activities of marker enzymes, lactate dehydrogenase (LDH) for cytosol, glutamate dehydrogenase (GDH) for mitochondrial matrix and ATPase insensitive to ouabain and $NaN_3$ and activable by calcium for myofilaments.

| Fraction | Medium for lysis | | |
|---|---|---|---|
| | Ringer (control) | Digitonin | Triton X-100 |
| Top | $S_R$: 10–20% LDH activity | $S_D$: 95% LDH activity ATPase | $S_T$: 100% LDH activity, 100% GDH activity ATPases |
| | broken cells + extracellular medium | cytosol + extracellular medium | mitochondria + cytosol + extracellular medium |
| Bottom | $B_R$: LDH activity GDH activity ATPases | $B_D$: no LDH activity 100% GDH activity ATPases: activated by Ca, inhibited by $NaN_3$ insensitive to ouabain | $B_T$: no LDH activity no GDH activity ATPases: activated by Ca insensitive to ouabain and $NaN_3$ |
| | whole cells | mitochondria + myofilaments | myofilaments |

*Compartmentation of the substrates of creatine kinase*

ATP, ADP, creatine phosphate and creatine contents were determined in the top and bottom fractions, as defined in Table I. The total recovery of metabolites, that is the sum of the supernatant and bottom fractions was compared to the metabolic content of the initial cell suspension after quenching by perchloric acid. The recovery of ATP and creatine phosphate was complete, whereas that of ADP was 98–103%. The determination of creatine was only possible on the fractions $B_R$ (whole cells), $S_D$ and $S_T$ (supernatant of lysis by either Triton or digitonin) as well as on the initial cell suspension. In the other fractions the amount of creatine was under the lower limit of detection in the fluorometric method used.

The 10–20% LDH activity observed in the Ringer supernatant fraction indicated the presence of broken cells. Due to these broken cells, ATP, creatine phosphate and ADP were released in the extracellular medium and titrated in the fraction $S_R$. These amounts varied from one cellular isolation to another and must be subtracted from the supernatants after lysis by either digitonin or Triton. The metabolite contents of the three main subcellular fractions, as defined in Table I, were determined for both lysis procedures. These data are summarized in Table II. The values were reported on the basis of the total cellular protein of the cell suspension.

TABLE II

SUBCELLULAR DISTRIBUTION OF ATP, ADP, CREATINE (OR Cr) AND CREATINE PHOSPHATE (OR CrP) IN RESTING OR BEATING HEART CELLS

For conditions used and denomination of the fractions, see Table I. The values are expressed in nmol/mg total protein ± SD. They are the mean of duplicate or triplicate determinations on seven cellular isolations. For each cellular isolation, resting and beating cells are compared. Intracellular content is obtained from $B_R$, myofilaments from $B_T$, mitochondria from $B_D - B_T$ and cytosol either from $S_D - S_R$ or $B_R - B_D$. n.d., not determined.

| Compartment | State of the cells | ATP | ADP | CrP | Cr |
|---|---|---|---|---|---|
| Intracellular | resting | 14 ±2 | 2.1±0.2 | 18.5±3 | 14.0±3 |
| | beating | 14 ±2 | 2.2±0.2 | 19.0±3 | 13.5±3 |
| Myofilaments | resting | 0 to 0.1 | 1.1±0.1 | 0 | n.d. |
| | beating | 0 to 0.6 | 1.1±0.06 | 0 | n.d. |
| Mitochondria | resting | 0.9±0.1 | 0.7±0.1 | 0 | n.d. |
| | beating | 0.8±0.1 | 0.8±0.1 | 0 | n.d. |
| Cytosol | resting | 13 ±2 | 0.05 to 0.25 [a] | 18.5±3 | 14.0±3 |
| | beating | 13 ±2 | 0.05 to 0.25 [a] | 19.0±3 | 13.5±3 |

[a] From bottom fractions; values obtained from supernatant were between −0.05 and +0.3.

Myofilament contents are determined directly from $B_T$. As indicated in Table II, only ADP is observed, the low ATP sometimes found is within the limits of experimental error; the metabolite contents of myofilaments in quiescent and beating cells are not significantly different ($p > 0.05$).

The metabolite content of mitochondria was determined from $B_D$ after subtraction of the myofilament contents $B_T$. In this compartment, the ATP/ADP ratio was between 1.3 and 1.0. When the ratio was compared for resting and beating cells from the same cellular isolation, the ratio was identical: a mean value 1.1 was observed. Creatine phosphate was never found in myofilaments, nor in mitochondria.

For the cytosolic compartment, the metabolite content may be obtained either from the supernates of digitonin fractionation after subtraction of the extracellular content ($S_D - S_R$) or by subtraction of mitochondrial plus myofilament of whole cells contents ($B_R - B_D$). The ATP contents determined by either way were similar, representing 90–92% of the total intracellular ATP. The differences in ADP content of control and digitonin lysates were very small, in the bottom fraction as well as in the supernates. As previously pointed out, ATP, creatine phosphate and ADP from broken cells is observed in the extracellular medium, so that a very slight variation of the concentration of metabolites during either the fast fractionation or the centrifugation and subsequent quenching of the control cells give rise to a great error on small differences such as those observed about ADP; for ATP, this possible error is not so important because the differences are large. To minimize the possible error introduced by slight variations of ADP it was better to consider the values obtained from bottom fractions, where the quenching by perchloric acid is obtained within 15–20 s after dilution by Ringer or lysis medium. The values for cytosolic ADP are very low: 0.05–0.25 nmol per mg of the initial cell suspension and thus the cytosolic ATP/ADP ratio is very hith (250–50). Moreover, the accuracy of the ADP determination is too low to permit observation of possible differences between resting and beating cells.

## Concluding remarks

The ATP, ADP, creatine and creatine phosphate content of heart have usually been determined after extraction from either whole organs or isolated cells [20,21,26,32,33]. In most studies, these metabolites were not determined directly in the subcellular compartments but rather computed from whole myocardium contents [32,33]. Mitochondrial ATP and ADP were extrapolated

from values observed on isolated mitochondria [33]. Myofilament compartment was evaluated after alcohol extract for firmly bound ADP to actin [9], and hypotheses were made about ATP or ADP binding to myosin during cardiac cycle [33]. Free cytosolic ADP was calculated assuming that creatine kinase reaction was at equilibrium in this compartment [5,32,33]. More recent determinations of cellular compartmentation in heart used fractionation of freeze-stopped tissues in non-aqueous media [34,35]. This method separated mitochondrial and extramitochondrial compartments but the hypothesis of creatine kinase equilibrium was necessary to estimate free ADP concentration. A high amount of ADP may be bound not only to myofilaments, but also to cytosolic proteins which are able to bind ADP [36]. The use of digitonin and Triton X-100 fast fractionation permitted the study on isolated cells of localization in the free main compartments: soluble cytosol, mitochondria and myofilaments.

The two most important observations were the following.

(a) In the myofilament compartment isolated by fast Triton X-100 fractionation, a high amount of ADP was observed. This bound ADP amounted to 1.1 nmol per mg total cellular proteins. No bound ATP was observed. A similar amount of bound ADP has been found in Triton-extracted cell ghosts [37]. This ADP could be bound to actin [9] and to myosin according to kinetic studies with isolated myosin, heavy meromyosin or myosin subfragment 1 [10,11]. Recent studies in rat muscle [6] have suggested that in resting muscle, no ADP was bound to myosin and that non-actin bound ADP increases in isotonically as well as isometrically working muscle. In the present study, the myofilament bound ADP did not increase when beating was induced by $BaCl_2$ or low KCl concentration. It is likely, that this ADP is bound to actin.

(b) Most of the cellular ATP was found in the soluble cytosolic compartment, and it is unlikely that ATP should be compartmented with a high concentration only in the intramitochondrial space. In the cytosolic compartment, the ATP/ADP ratio is very high. By taking into account the cytosolic pH of 7.1 determined by NMR [38] and the total and free Mg concentrations [39], a value comprised between $2 \cdot 10^{-9}$ and $0.5 \cdot 10^{-9}$ M may be estimated for the ratio

$$\frac{[\text{creatine phosphate}][\text{ADP Mg}][H^+]}{[\text{ATP Mg}][\text{creatine}]}$$

in this compartment. This value is near the equilibrium constant determined by Lawson and Veech [40] and indicates that the hypothesis that the creatine kinase reaction is at or near equilibrium in the cytosol of heart cells is justified. The low level of ADP in either beating or resting cells, as well as the non-synchroneous beating of the cells does not permit observation of possible differences between them.

A preliminary account of a portion of this work has been presented before [41].

## Acknowledgements

This work has been supported by the Ministère de la Recherche et de la Technologie (Biologie Moléculaire 82.V.1267), by the Centre National de la Recherche Scientifique (action thèmatique conversion de l'énergie dans les membranes biologiques) and by the Institut National de la Santé et de la Recherche Médicale (Unité 241). We wish to thank Dr. G. Vassort, Director of the laboratory, and all our colleagues for stimulating discussions, especially D. Angelini for secretarial assistance.

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