NMR-Invisible ATP in Rat Heart and Its Change in Ischemia

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Received for publication, February 15, 1988

The subcellular compartmentalization of adenosine 5'-triphosphate (ATP) in isolated perfused rat heart and its relation to energy depletion in ischemia were examined by 31P nuclear magnetic resonance (31P-NMR) spectroscopy and chemical analyses. The signal intensities of the β-phosphate of ATP and creatine phosphate in the 31P-NMR were standardized by the intracellular volume ratio measured with 23Na-NMR to determine the actual content of each. During aerobic perfusion the ATP content determined by NMR (13.7 ± 2.2 μmol/g dry weight) was significantly lower than that found by chemical analysis (22.4 ± 0.7 μmol/g dry weight), while the creatine phosphate contents determined by the two methods were the same. During ischemia at 33°C, the signal of the β-phosphate of ATP in the 31P-NMR spectrum decreased progressively, disappearing completely after 16 min. But at this time 5.7 ± 1.7 μmol/g dry weight of myocardial ATP was still detected by chemical analysis. These results indicated that there were two different compartments of intracellular ATP in the heart, only one of which is detectable by 31P-NMR spectroscopy, and that during ischemia the ATP that is detectable, which seems to be the free ATP in the cytosol, decreased more rapidly than the ATP in the other compartment.

The adenosine 5'-triphosphate (ATP) content of the myocardium decreases during ischemia, resulting in myocardial dysfunction on reperfusion (1, 2). The viability of the heart has been assessed by measurement of the myocardial ATP content (3, 4). Although the correlation between the myocardial ATP level and cardiac function is still controversial (5–7), it has been generally accepted that when the myocardial ATP level decreases to 20 or 30% of the original level during ischemia, functional recovery is impossible (3, 8). This absence of functional recovery in the presence of residual ATP has been explained by postulating that the ATP may be exhausted in some intracellular compartments when the ATP content of the myocardium as a whole decreases below a certain level during ischemia (9, 10). However, there has been no report of studies on this possible subcellular compartmentalization of ATP in the myocardium related with ischemia.

Recently, 31P nuclear magnetic resonance (31P-NMR) has been used to observe changes in the levels of high energy phosphate compounds in the perfused heart. However, it has been shown that immobile phosphate groups, such as phospholipids in the cell membrane and ADP bound to actin filaments, cannot be detected by 31P-NMR spectroscopy because of broadening of the signals (11, 12). The 31P-NMR signals of ATP in mitochondria might also be broadened to invisibility by the presence of paramagnetic ions (13). These observations by 31P-NMR spectroscopy suggest that both the bound form of ATP and mitochondrial ATP in the heart are likely to be silent on 31P-NMR spectroscopy. Therefore, we used a combination of 31P-NMR spectroscopy and chemical analysis, which detects all phosphate compounds in the tissue, to demonstrate the existence of the NMR-invisible ATP in perfused heart.

The direct comparison of data obtained in 31P-NMR analyses with the chemical analyses is difficult because the former method gives only the relative content of high energy phosphate in a tissue. Therefore, in this study we standardized the 31P-NMR data, using 23Na-NMR spectroscopy to measure the intracellular volume ratio of the heart, and then compared the data obtained by 31P-NMR spectroscopy with those obtained by chemical analysis. Using this combined approach, we found that about 40% of the total ATP content of rat heart was NMR-invisible, and that this form of ATP decreased much more slowly than the NMR signal of ATP during ischemia.

MATERIALS AND METHODS

Isolated Heart Preparations—Male Sprague-Dawley rats, weighing about 180 g, were used after starvation for 24 h. They were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and their hearts were rapidly removed. The aorta was promptly cannulated, and perfused by the Langendorff technique. The perfused heart was transferred to a glass tube (15 mm in diameter), and the perfusate was continuously aspirated, keeping the heart submerged in the fluid. The perfusate consisted of (in

1This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.
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Abbreviations: ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography.
Measurement of the Ratio of the Intracellular Volume to the Dry Weight of Hearts—As described above, NMR measurements gave the content of ATP and creatine phosphate in \( \mu \text{mol} \) per ml of intracellular volume of the heart. For comparison of these values with those obtained by chemical analyses, the contents must be expressed in \( \mu \text{mol} \) per g dry weight. The ratio of the dry weight of the heart (in g) to the intracellular volume of the heart (in ml) was obtained in a separate experiment with sucrose as a marker of the extracellular space (17). In this experiment, rat hearts were perfused in the same fashion, except that the medium contained 1.5 mM sucrose and glucose was omitted. The hearts were perfused for 30 to 120 min to allow the sucrose to equilibrate with all the extracellular space. Then the hearts were blotted, freeze-clamped, and lyophilized (n=5).

The intracellular volume (ml/g dry weight of heart): \( V_{IC} \) was obtained from the difference between the total volume (ml/g dry weight): \( V_T \) and the extracellular volume (ml/g dry weight): \( V_{EC} \).

\[
V_{IC} = V_T - V_{EC}
\]

(1)

\( V_T \) was determined according to Eq. 2 by measuring the wet weight (g/g dry weight), and specific gravity of the perfused heart by floating a small piece of the heart in sucrose solutions of various concentrations.

\[
V_T = \frac{\text{wet weight, g/g dry weight}}{\text{specific gravity}}
\]

(2)

\( V_{EC} \) was obtained by Eq. 3.

\[
V_{EC} = \frac{\text{sucrose content of heart/g dry weight}}{\text{sucrose content in 1 ml of coronary effluent}}
\]

(3)

The sucrose content of a perchloric acid extract of lyophilized heart and the content in the coronary effluent, which was equal to that of the perfusate, were determined by measuring increase in glucose in treatment with sucrose [EC 3.2.1.48] by the method of Furuya et al. (18).

RESULTS

ATP and Creatine Phosphate Contents of the Myocardium—The contents of ATP and creatine phosphate of the hearts during aerobic perfusion were determined by chemical analyses to be 22.4 ± 0.71 (mean ± S.D.) and 22.9 ± 5.12 \( \mu \text{mol/g dry weight} \) (n=9), respectively. \( ^{31}\text{P}-\text{NMR spectroscopy} \) was also performed during aerobic perfusion, and Fig. 1 shows a representative spectrum. The values for the ATP and creatine phosphate contents obtained by a combination of \( ^{31}\text{P}-\text{NMR} \) and \( ^{23}\text{Na}-\text{NMR} \) spectroscopy were 3.38 ± 0.53 and 5.61 ± 1.41 \( \mu \text{mol/ml of intracellular volume} \), respectively (n=5) (Table I).

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Results on the ratios of the intracellular volume to the dry weight of perfused hearts at three points during aerobic perfusion (n=5) are shown in Fig. 2. Although values for the ratio of the intracellular or extracellular volume to the total tissue volume were somewhat scattered, the ratio of the dry weight (g) to the intracellular volume (ml) was almost constant between 30 and 120 min of perfusion, being 0.246±0.037 g dry weight per ml of intracellular volume. This value (0.246 g dry weight/ml Vc) was used to calculate the specific contents of ATP and creatine phosphate from NMR data. The values obtained during aerobic perfusion were 13.7±2.2 and 22.8±5.8 μmol/g dry weight, respectively (Table I). The value for the ATP content was significantly lower than that obtained by chemical analysis (p<0.01), but the value for creatine phosphate was similar to that determined by chemical analysis (Fig. 3).

Changes in the ATP Level during Ischemia and Reperfusion—Figure 4 shows the consecutive 31P-NMR spectra recorded every 2 min during the control period before ischemia (top 2 traces), and during ischemia (15 traces), and subsequent reperfusion (bottom 4 traces). During ischemia, the signal of creatine phosphate (at 0 ppm) rapidly decreased and disappeared after 5 min. The signal due to the β-phosphate of ATP (at -16 ppm) decreased more slowly, disappearing after 16 min. The signal of γ-ATP and β-ADP (at -2.5 ppm) diminished like that of β-ATP. The peak of α-ATP and α-ADP (at -7.5 ppm), which fused with the peak of NAD, decreased, but a broad signal remained for longer than the signals of β-ATP and γ-ATP. During reperfusion, the signal of creatine phosphate increased rapidly to a supranormal level, but recovery of the signals of ATP was slow.

The changes in the ATP level in the heart during ischemia measured by NMR analysis (n=5) and chemical analysis (n=3) are shown in Fig. 5. At 0 time, the value obtained by chemical analysis was 1.6 times that determined from the 31P-NMR spectrum, as described above. The ATP content decreased progressively during ischemia, and after 16 min no β-phosphate of ATP was detected by 31P-NMR spectroscopy. The complete disappearance of the 31P-NMR signal of the β-phosphate of ATP was confirmed by the spectrum shown in Fig. 6. In Fig. 6, panels (A) and (B) show the accumulated 31P-NMR spectra of 5 hearts, each obtained with an accumulation time of 1 min, during aerobic perfusion and after 16 min of ischemia, respectively. Although after 16 min no signal of the β-phosphate of ATP was detectable, 5.7±1.7 μmol ATP per g dry weight of heart, corresponding to 25% of the original content, was found by chemical analysis.

When the heart was reperfused after 18 min of ischemia, the ATP content was partially restored. After reperfusion for 5 min, its content was found to be 3.2 μmol/g dry weight (n=1) by 31P-NMR measurement, and 14.6 μmol/g dry weight (n=1) by chemical analysis.

![Fig. 2. The relationships of the dry weight, the intracellular volume, and the total tissue volume of the heart during aerobic perfusion. Closed triangles (▲) show ratios of g dry weight to ml of intracellular volume, closed circles (●) show those of ml of intracellular volume to ml of total tissue volume, and open circles (○) show those of ml of extracellular volume to ml of total tissue volume. The right panel shows the mean±S.D. of each parameter throughout the perfusion (n=5).](https://academic.oup.com/jb/article-abstract/104/1/35/799977)

### TABLE I. Contents of ATP and creatine phosphate in hearts measured by NMR spectroscopy during aerobic perfusion.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Ratio of Vc</th>
<th>Signal of 31P-NMR</th>
<th>μmol per ml Vc</th>
<th>μmol per g dw</th>
<th>Signal of 31P-NMR</th>
<th>μmol per ml Vc</th>
<th>μmol per g dw</th>
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<tr>
<td>1</td>
<td>0.404</td>
<td>0.953</td>
<td>2.36</td>
<td>9.59</td>
<td>1.39</td>
<td>3.44</td>
<td>13.9</td>
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<td>2</td>
<td>0.230</td>
<td>0.796</td>
<td>3.46</td>
<td>14.1</td>
<td>1.74</td>
<td>7.57</td>
<td>30.8</td>
</tr>
<tr>
<td>3</td>
<td>0.247</td>
<td>0.879</td>
<td>3.56</td>
<td>14.5</td>
<td>1.42</td>
<td>5.75</td>
<td>23.4</td>
</tr>
<tr>
<td>4</td>
<td>0.221</td>
<td>0.899</td>
<td>3.89</td>
<td>15.8</td>
<td>1.50</td>
<td>6.48</td>
<td>28.3</td>
</tr>
<tr>
<td>5</td>
<td>0.257</td>
<td>0.928</td>
<td>3.81</td>
<td>14.7</td>
<td>1.24</td>
<td>4.83</td>
<td>19.6</td>
</tr>
<tr>
<td>Mean±S.D.</td>
<td>0.38±0.53</td>
<td>3.8±1.25</td>
<td>13.7±2.15</td>
<td>5.6±1.41</td>
<td>22.8±5.75</td>
<td>6.5±1.75</td>
<td>22.8±5.75</td>
</tr>
</tbody>
</table>

*Ratio of the intracellular volume of the heart in the observation field of the NMR, which was determined by 23Na-NMR spectroscopy as described in "MATERIALS AND METHODS." Signal intensity of the β-phosphate of ATP or creatine phosphate in arbitrary units. μmol per ml Vc: μmol per ml of intracellular volume, calculated by dividing the signal intensity of the 31P-NMR by the ratio of Vc. μmol per g dry weight. Values were converted from μmol per ml Vc to μmol per g dw using the ratio of 0.246 g dw/ml Vc.

Vol. 104, No. 1, 1988
DISCUSSION

Continuous and noninvasive measurement of high energy phosphates in the heart is possible by $^{31}$P-NMR spectroscopy. However, this method is known not to detect all phosphate compounds in the tissue. For example, phospholipids in the cell membrane (12) and ADP bound to actin filaments (11, 19) cannot be detected by $^{31}$P-NMR spectroscopy, because their signals are broadened to invisibility due to their extremely short relaxation time in NMR. Thus, phosphate groups whose free motion is restricted by binding to membranes or proteins are considered not to be detectable by $^{31}$P-NMR. In heart, there are many kinds of proteins and enzymes and above all large amounts of myofibrils in the myocardium, which are supposed to bind significant amounts of ATP (20). This type of bound-ATP may also not be detectable by $^{31}$P-NMR spectroscopy of perfused heart. Furthermore, mitochondrial ATP may also not be detectable by $^{31}$P-NMR spectroscopy in the perfused heart. Ogawa et al. (21) described that a mitochondrial suspension did not show the $^{31}$P-NMR signal of $\beta$-phosphate of ATP unless EDTA was added, or the mitochondria were extracted with perchloric acid. Paramagnetic metal ions such as manganese that are reported to be abundant in mitochondria (22) may broaden the signals of mitochondrial ATP to invisibility (13).

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In this study the myocardial ATP content determined by $^{31}$P-NMR spectroscopy was converted to an absolute value by use of the intracellular volume ratio measured by $^{23}$Na-NMR spectroscopy. We determined the intracellular volume ratio by $^{31}$Na-NMR spectroscopy based upon the fact that the intracellular sodium concentration is very much lower than that in the extracellular space under aerobic conditions (23, 24). The value for the ATP content of the heart during aerobic perfusion obtained by NMR was about 60% of that determined by chemical analysis, while the creatine phosphate content obtained by NMR was similar to that determined by chemical analysis. Since creatine phosphate is reported to be present only in the cytoplasm (25, 26) and to move freely as a shuttle transporting high energy phosphate bond (27), all the creatine phosphate present should be detectable by $^{31}$P-NMR spectroscopy.

We found that about 40% of the total ATP was not detectable by $^{31}$P-NMR. Since the mitochondrial volume in the myocardial cells has been estimated to be about 36% of the cell volume (28), the ratio of mitochondrial ATP to the total seems to be around this value. The myosin-bound ATP was calculated to be about 4% of total ATP based on the reported value of myosin content in myocardium (29). The ATP that was not detectable by NMR is likely to include these mitochondrial ATP and protein-bound ATP, while the other portion of about 60% of the total ATP that was detectable by NMR was probably free ATP in cytosol.

During ischemia, the signal of the $\beta$-phosphate of ATP observed by $^{31}$P-NMR spectroscopy in the heart disappeared after 16 min. But at this time about 25% of the original ATP content was still detectable by chemical analysis. These results suggest that during ischemia the free ATP in the cytosol is depleted earlier than the protein-bound or mitochondrial ATP. Previously we found that during ischemia mitochondrial ATP decreases more slowly than total ATP in liver tissue (14, 30). Mitochondrial ATP may not decrease as quickly as that in the cytosol, because when oxidative phosphorylation stops during ischemia ATP-$$^\text{ADP3-}$$ in the mitochondria may not be exchanged with ADP-$$^\text{ADP3-}$$ through the inner mitochondrial membrane due to imbalance of the anionic charge.

The relationship between the cardiac contractile function and its ATP level is still controversial. Reibel and Rovetto (6) and Nishioka and Jarmakani (31) reported a linear correlation between the myocardial ATP content and cardiac function. Taegtmeyer et al. (7) and Rosenkranz et al. (32), however, observed no correlation between the two. Our results indicated that there are at least two different forms of myocardial ATP, which may play different roles in protection of cells against injuries. Therefore, the changes in the respective forms of cellular ATP, not the total content, should be considered in relation to function.

This work showed that there are two forms of ATP in rat heart: one is detectable by $^{31}$P-NMR spectroscopy and the other is not. From the characteristics of the $^{31}$P-NMR, the former seems to be free ATP in the cytosol and the latter to be protein-bound and mitochondrial ATP. During ischemia, the free ATP in the cytosol seemed to be depleted more rapidly than the other form of ATP.

REFERENCES