Localization of catalytic and regulatory subunits of cyclic AMP-dependent protein kinases in mitochondria from various rat tissues

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INTRODUCTION

The presence of cyclic AMP-dependent protein kinases in mammalian mitochondria is still a matter of controversy. An influence of cyclic AMP, or of hormones acting by increasing intracellular cyclic AMP, on mitochondrial functions has been described repeatedly, but has been questioned also. Stimulation of respiration in liver mitochondria from glucagon-treated rats [1] was considered as a possible artifact of the mitochondrial preparation [2,3]. A report on a cyclic-AMP-triggered release of Ca²⁺ from kidney, liver and heart mitochondria [4] was retracted 2 years later by that author following criticism by various groups [5]. Increased progesterone secretion after luteinizing hormone stimulation of bovine corpora lutea was suggested to result from cyclic AMP-dependent protein kinase-catalysed phosphorylation of the mitochondrial side-chain-cleavage cytochrome P-450 [6]. However, other authors could not achieve phosphorylation of purified side-chain-cleavage cytochrome P-450 enzyme with the catalytic subunit of cyclic AMP-dependent protein kinases [7].

A relatively high activity of cyclic AMP-dependent protein kinases has been reported in mitochondria from antral follicles and corpora lutea of pig ovaries [8], but the purity of the mitochondrial fraction has been questioned [9]. Only negligible cyclic AMP-dependent protein kinase activity was measured in rat liver mitochondria by Chen & Walsh [10], and Vardanis [11] did not detect any such protein kinase activity in carefully isolated mitochondria from mouse liver or mouse and bovine heart. On the other hand, Kleitke et al. [12] and Henriksson & Jergil [13] measured a small but significant amount of cyclic AMP-dependent protein kinase activity in rat liver mitochondria, and Burgess & Yamada [14] detected this protein kinase activity in mitochondria from bovine heart muscle. Hasegawa [15] described cyclic AMP-dependent protein kinase activity in rat adrenal mitochondria, but Deviller et al. [16] did not detect any such protein kinase activity in bovine adrenal cells.

The few investigations on the submitochondrial localization of cyclic AMP-dependent protein kinase also led to different results. In rat liver, 80–90% of the enzyme activity was localized in the intermembrane space [13]; in bovine heart muscle most of the activity was found to be associated with the matrix region [14]. An immunocytochemical study of the localization of the regulatory subunit RII of cyclic AMP-dependent protein kinases in sperm flagellae suggested the presence of type II cyclic AMP-dependent protein kinase at the outer membrane of the mitochondria [17].

In the present report we demonstrate, with antibodies directed against the catalytic (C) and regulatory (RI and RII) subunits of type I and type II cyclic AMP-dependent protein kinases, labelling in situ of mitochondria from various rat tissues which exceeds the labelling density observed in other cell compartments and which is localized at the inner membrane/matrix space. The results are supported by immunochemical and partly also by enzyme-activity determinations of cyclic AMP-dependent protein kinases in mitochondria and mitochondrial subfractions prepared from broken tissues.

MATERIALS AND METHODS

Materials

Kemptide, histones (IIA-S), cytochrome c, benzanilide, 1,10-phenanthroline and chloromethane ("TPCK"), aprotinin, Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, Lubrol and RNA were from Sigma (Deisenhofen, Germany). NADH, oxalacetate, phenylmethylene sulfonamide fluoride and Protein A were from Boehringer (Mannheim, Germany). Leupeptin, antipain, chymostatin and pepstatin were from The Peptide

Abbreviations used: C, catalytic subunit of the cyclic AMP-dependent protein kinases; RI or RII, regulatory subunits of cyclic AMP-dependent protein kinases type I or type II respectively.

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Institute (Osaka, Japan). Glutaraldehyde was from Serva (Heidelberg, Germany), dithioerythritol from Biomol (Ilvesheim, Germany), Ficoll-400 from Pharmacia (Uppsala, Sweden), polyvinylpyrrolidone from Roth (Karlsruhe, Germany), BSA from Behringwerke AG (Marburg, Germany), and pentobarbital from Ceva (Düsseldorf, Germany). Cyclic [3H]AMP (31 Ci/mmole) was from New England Nuclear (Drizeich, Germany), and 131I-Protein A and 14C-labelled marker proteins were from Amersham (Braunschweig, Germany). Protein A labelled with 15 nm gold particles was purchased from Jansen (Beerse, Belgium), and alkaline phosphatase-conjugated goat anti-rabbit IgG was from Sottocasa et al. (Karlsruhe, Germany), BSA from Serva (Heidelberg, Germany), and carbonic anhydrase (30000).

For localization of cyclic AMP-dependent protein kinase subunits, a protein A-gold method [31] was applied as described previously [23]. Antisera were used at a dilution of 1:2000 (anti-C) and 1:50 (anti-RI, anti-RII), and Protein A conjugated with 15 nm gold particles at a dilution of 1:10. The immunolabelled thin sections were stained with uranyl acetate and lead citrate, and observed and photographed in a Zeiss EM 9 S-2 electron microscope.

Quantification of the immunoreaction was performed by counting the number of gold particles in predetermined areas of the micrographs at a final magnification of ×22 500. The areas were determined in mitochondria by the point-counting method [32], and in other cell compartments by randomly superimposed transparent standards of 1 μm².

Cyclic AMP-dependent protein kinase activity

This was determined under conditions established for the measurement of the enzyme in particulate cell fractions [33]. Samples were pretreated with 2% (w/v) Triton X-100 for 5 min at 30°C. Incubation was performed up to 6 min at 30°C with 15–20 μg of protein in 20 mM-Hepes buffer, pH 7.8, containing 10 mM-magnesium acetate, 1 mM-dithioerythritol, 25 nmol of [32P]ATP (1 μCi), 1.8 μM cyclic AMP and 145 μg of mixed histones or 0.1 mM-Kemptide, in a final volume of 110 μl. The radioactivity incorporated into histones was determined as in [34], and that incorporated into Kemptide as in [35]. Additional determinations in the presence of saturating amounts of the heat-stable inhibitor of cyclic AMP-dependent protein kinases served to discriminate the cyclic AMP-dependent protein kinase activity from other kinase activities. Controls for unspecific labelling were performed with heat-denatured (5 min, 95°C) tissue samples.

Enzyme-linked immunosorbent assay

A competitive e.l.i.s.a. of the C-subunit was performed as described previously [36]. Purified C-subunit (4 ng/well) was adsorbed on the plastic surface of microtitre plate wells, and binding of antibodies from C-antisera, diluted 1:4000, in the absence and presence of various dilutions of tissue extracts was detected by the enzyme reaction of alkaline phosphatase-coupled anti-(rabbit IgG) antibody. The tissue samples had been pretreated with 6 mM-urea and sonication [36].

Immunoblotting

Immunoblotting was performed essentially as described by Towbin et al. [37]. Mitochondria and mitochondrial subfractions were separated by SDS/PAGE and transferred on to nitrocellulose paper by electroblotting. The C- and R-subunits of cyclic AMP-dependent protein kinase were detected by incubation with specific antisera diluted 1:1000 (anti-C) or 1:200 (anti-RI), followed by incubation with 125I-Protein A (1.15 μCi/10 ml) and radiography. 14C-labelled M₆ markers were phosphorylase b (95 500), BSA (69 000), ovalbumin (46 000) and carbonic anhydrase (30 000).

Other methods

Amylase was measured as described in [38], monoamine oxidase as in [39], and succinic dehydrogenase and cytochrome c oxidase as in [40]. Malate dehydrogenase was determined with 190 μM-NADH and 470 μM-oxalacetate as described in [28], and lactate dehydrogenase with 350 μM-NADH and 10 mM-pyruvate.
Mitochondrial cyclic AMP-dependent protein kinases

Fig. 1. Immuno-gold localization of the cyclic AMP-dependent protein kinase catalytic subunit in rat liver, heart and kidney cells

Lowicryl-K4M-embedded thin sections from glutaraldehyde-fixed rat liver, heart and kidney tissue were reacted with anti-(C-subunit) serum, followed by incubation with 15 nm-gold-labelled Protein A. As shown in the left panel, a high C-subunit immunoreactivity occurs in mitochondria (M) of hepatocytes (1), cardiomyocytes (2) and kidney cells from the pars convoluta (3). Immunolabelling is also seen in the nucleus (N), the endoplasmic reticulum (ER) and in myofibrils (A, A-band; I, I-band; arrow, Z-disc) of the cardiomyocytes. No significant immunoreaction is seen in the intercellular space (IS) or in the images obtained when pre-immune serum was used instead of anti-(C-subunit) serum (right panel). Magnification x 31 500. Bar represents 0.5 μm.

RESULTS

Immunocytochemical localization and quantification of C-, RI- and RII-subunits in mitochondria from various rat tissues

Immuno-gold electron microscopy revealed a remarkably high immunoreactivity of mitochondria from various rat tissues with antiserum against the catalytic subunit of cyclic AMP-dependent protein kinases (Fig. 1, left panel; Table 1). Omission of the antiserum, preincubation of the Protein A-gold solution with anti-C (or anti-R) serum or preincubation of thin sections with unlabelled protein A abolished the immunolabelling (results not shown). This was also the case after replacement of antiserum with preimmune serum (Fig. 1, right panel).
Table 1. Quantification of C-subunit immunoreactivity in mitochondria, cytoplasm and nuclei of cells from various rat tissues

The number of gold particles in mitochondria, cytoplasm and nuclei was counted in n cells analysed in tissues taken from three rats. In each cell 10 mitochondria or 3 randomly chosen areas of 1 μm² each (cytoplasm, nuclei) were evaluated. The values are means ± S.E.M. The labelling density in the intercellular space was 0-1 gold particles/μm². ‘Cytoplasm’ includes cytoplasmic structures with the exception of mitochondria and granules.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell compartment</th>
<th>Mitochondria</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte</td>
<td></td>
<td>49.5 ± 2.7</td>
<td>19.1 ± 1.1</td>
<td>20.3 ± 1.1</td>
<td>18</td>
</tr>
<tr>
<td>Pancreatic acinar cell</td>
<td></td>
<td>36.7 ± 5.2</td>
<td>8.7 ± 1.3</td>
<td>7.8 ± 0.7</td>
<td>8</td>
</tr>
<tr>
<td>Parotid acinar cell</td>
<td></td>
<td>47.0 ± 6.0</td>
<td>11.5 ± 2.1</td>
<td>10.2 ± 1.3</td>
<td>39</td>
</tr>
<tr>
<td>Cardiomyocyte</td>
<td></td>
<td>39.2 ± 7.3</td>
<td>29.5 ± 5.1</td>
<td>15.1 ± 2.8</td>
<td>5</td>
</tr>
<tr>
<td>Skeletal-muscle myocyte</td>
<td></td>
<td>42.8 ± 4.1</td>
<td>23.4 ± 1.6</td>
<td>11.9 ± 0.6</td>
<td>10</td>
</tr>
<tr>
<td>Kidney cell (pars convoluta)</td>
<td></td>
<td>37.8 ± 4.2</td>
<td>13.5 ± 2.0</td>
<td>11.7 ± 1.2</td>
<td>10</td>
</tr>
<tr>
<td>Nerve cell (cortex)</td>
<td></td>
<td>59.6 ± 7.9</td>
<td>22.3 ± 3.9</td>
<td>22.4 ± 3.8</td>
<td>13</td>
</tr>
</tbody>
</table>

Fig. 2. Immuno-gold localization of catalytic and regulatory subunits of cyclic AMP-dependent protein kinases in rat mitochondria

Immunolabelling is seen by reaction with specific antisera against the C-subunit (panels 1a, 1b, 2a, 3a), as well as against the RI-subunit (panels 1c, 2b, 3b) and the RII-subunit (panels 1d, 2c, 3c) in cristae-type mitochondria from hepatocytes (panels 1a, 1c, 1d), exocrine pancreatic cells (panels 2a–2c) and cardiomyocytes (panels 3a–3c) as well as in tubular-type mitochondria from Sertoli cells (panel 1b). Most of the gold particles appear at the inner membrane (bright) directed to the matrix (dark). Magnification: panels 1a, 2a, 3a–3c, × 54000; other panels, × 66400. Bar represents 0.2 μm.
Cytoplasm and cell nuclei showed in most tissues a quantitatively similar labelling density, which amounted to 20-40% of that calculated for the mitochondria (Table 1). Myocytes from heart and skeletal muscle exhibited a somewhat higher labelling density in the cytoplasm than in the nucleus. This was due to C-subunit immunoreactivity at the actomyosin of myofilaments (Fig. 1, row 2).

Immunolabelling of hepatocytes, pancreatic acinar cells and cardiomyocytes with antisera against cyclic AMP-dependent protein kinase regulatory subunits showed again the highest labelling density in the mitochondria and in the cell nuclei (Table 2). In hepatocytes, the immunoreactivity obtained in mitochondria with the anti-RI and -RII antisera was equally high (Fig. 2, panels 1c and 1d; Table 2); relatively more RII than RI immunoreactivity was found in the cytoplasm and in the cell nuclei (Table 2). The pancreatic acinar cells and the cardiomyocytes showed in the mitochondria (Fig. 2, panels 2b, 2c, 3b, 3c; Table 2), as well as in the nuclei and cytoplasm (Table 2), predominantly RI immunoreactivity.

In all tissues examined, the majority of gold particles indicating C- or R-subunits found in mitochondria appeared to be localized at the inner side of the inner membrane. This was the case in mitochondria of the cristae-type as well as of the tubular-type (Fig. 2).

![Image](image_url)

**Fig. 3.** Electron micrograph of a mitochondrial cell fraction purified from rat parotid glands

A mitochondrial cell fraction was prepared as described in ref. [25] and Spurr-embedded thin sections of glutaraldehyde/OsO4-fixed samples were examined in the electron microscope. Magnification ×6000. Bar represents 2 μm.

**Table 2. Quantification of RI- and RII-subunit immunoreactivity in mitochondria, cytoplasm and nuclei of rat liver, pancreas and heart cells**

Labelling densities were determined as described in the legend of Table 1. The three tissues were analysed in parallel.

<table>
<thead>
<tr>
<th>Immunoreactivity</th>
<th>Cell type</th>
<th>Cell compartment</th>
<th>Mitochondria</th>
<th>Cytoplasm</th>
<th>Nuclei</th>
<th>Intercellular</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-RI</td>
<td>Hepatocyte</td>
<td></td>
<td>18.8±1.2</td>
<td>8.5±0.8</td>
<td>3.7±0.3</td>
<td>1.1±0.3</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Pancreatic acinar</td>
<td></td>
<td>23.0±1.7</td>
<td>11.1±1.0</td>
<td>5.1±0.6</td>
<td>0.3±0.2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Cardiomyocyte</td>
<td></td>
<td>21.6±0.9</td>
<td>12.1±0.7</td>
<td>3.3±0.3</td>
<td>0.4±0.1</td>
<td>20</td>
</tr>
<tr>
<td>Anti-RII</td>
<td>Hepatocyte</td>
<td></td>
<td>22.9±2.4</td>
<td>16.8±1.7</td>
<td>12.7±2.1</td>
<td>5.0±0.7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Pancreatic acinar</td>
<td></td>
<td>6.6±1.5</td>
<td>4.4±0.9</td>
<td>3.6±0.5</td>
<td>4.4±0.8</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Cardiomyocyte</td>
<td></td>
<td>4.3±0.8</td>
<td>2.6±0.4</td>
<td>1.2±0.2</td>
<td>1.2±0.2</td>
<td>5</td>
</tr>
</tbody>
</table>

**Fig. 4. E.l.i.s.a. determination of cyclic AMP-dependent protein kinase C-subunit with isolated mitochondria and with a 1200 g supernatant fraction from rat parotid glands**

Binding of anti-C-subunit antibodies to purified C-subunit coated on to the plastic surface of microtitre plates (4 ng/well) is competed for by various dilutions of protein from isolated parotid mitochondria (○) or from a parotid 1200 g supernatant (●). About 4-fold less mitochondrial than supernatant protein gives the same inhibition of antibody binding. A/Ao is the absorbance measured in the presence of competing antigen relative to that measured in the absence of competing antigen.

**Quantification of C-subunit by e.l.i.s.a. in isolated parotid-gland mitochondria**

A mitochondrial fraction was isolated from parotid glands as described in the Materials and methods section. It was almost free from other organelles, in particular from the similarly sedimenting secretory granules or from microsomal material as revealed by marker-enzyme analysis [43] and by morphological examination (Fig. 3).

Quantification by competitive e.l.i.s.a. of the amount of C subunits present in the isolated mitochondria compared with
that present in a crude 1200 g supernatant revealed a 4-fold higher concentration of C-subunit in the mitochondria (Fig. 4). This result agrees remarkably well with the result of the immunocytochemical determination (Table 1).

**Immunolocalization of C- and R-subunits in subfractions from rat liver mitochondria**

Mitochondrial subfractions were prepared from isolated liver mitochondria by the use of digitonin and Lubrol and characterized by marker-enzyme analysis (Table 3).

Immunoblotting indicated an enrichment of C-subunits in mitoplasts and an almost complete absence of C-subunits in the residual mitochondrial fraction containing outer membranes and intermembrane space (Fig. 5, left panel). This is in line with our immunocytochemical findings (Figs. 1 and 2). The inner membrane and matrix fractions both showed a similarly high C-subunit immunoreactivity (Fig. 5). However, in different experiments the distribution of C-subunit immunoreactivity between these two fractions varied to some extent, even though the quality of the separation as judged by marker-enzyme analysis was similar. The average specific immunoreactivity of C-subunit in both fractions as calculated from the radioactivity of bound 125I-Protein A in five separate experiments revealed almost equal amounts in these two fractions (Table 3). A calculation which takes into account the proportion of total mitochondrial protein in the sub mitochondrial fractions [27] indicated that about 80% of the mitochondrial cyclic AMP-dependent protein kinase C-subunits were found in the matrix fraction (Table 3).

Detection of the RI-subunit in submitochondrial fractions by immunoblotting is shown in Fig. 5, right panel. Even in the presence of various proteinase inhibitors a considerable portion of the RI subunits (Mr, 49000) appeared to be degraded to the known [44,45] degradation product of Mr, 38000. As with the C-subunit, RI-subunit immunoreactivity was enriched in the mitoplasts and was almost absent in an outer membrane/intermembrane fraction. However, in contrast with the C-subunit, the RI-subunit seemed to be exclusively localized in the inner membrane fraction of mitoplasts (Fig. 5, right panel lanes d, e). In none of five independent experiments was RI-subunit immunoreactivity observed in the matrix region.

Attempts to localize RII-subunit immunoreactivity in submitochondrial fractions by immunoblotting were so far unsuccessful. The very weak reaction which could be sometimes observed, however, suggested its presence also in the matrix region.

**Specific activity of cyclic AMP-dependent protein kinase in mitochondria**

As shown and discussed previously by us [33], various factors interfere with the determination of cyclic AMP-dependent protein kinase activities in parotid-gland particulate cell fractions. The determination could be improved by increasing the ATP concentration in the assay and by pretreatment of samples with Triton X-100 [33]. In addition, use of Kemptide as a more specific substrate than the frequently used histones considerably enhanced the cyclic AMP-dependent protein kinase activity measured in isolated parotid mitochondria. Nevertheless, even under these conditions the kinase activity amounted only to less than 10% of the activity measured in the parotid cytosol (results not shown).

In contrast, liver mitochondria, which could be isolated by a much faster procedure than was possible for parotid mitochondria, exhibited a relatively high kinase activity under the same experimental protocol (Table 4). This activity was not

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**Table 3. Distribution of marker-enzyme activities and of C-subunit immunoreactivity in mitochondrial subfractions**

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Mitochondria</th>
<th>Mitoplasts</th>
<th>Outer m./interm.</th>
<th>Inner m.</th>
<th>Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH (cytosol)</td>
<td>142</td>
<td>9</td>
<td>230</td>
<td>6</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sp. activity</td>
<td>0.045</td>
<td>0.004</td>
<td>0.087</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>RSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAO (outer m.)</td>
<td>5</td>
<td>3</td>
<td>18</td>
<td>2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sp. activity</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyt. c ox. (inner m.)</td>
<td>287</td>
<td>424</td>
<td>44</td>
<td>233</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sp. activity</td>
<td>8</td>
<td></td>
<td></td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDH (inner m.)</td>
<td>127</td>
<td>129</td>
<td>6</td>
<td>120</td>
<td>4</td>
</tr>
<tr>
<td>Sp. activity</td>
<td>2</td>
<td></td>
<td></td>
<td>90</td>
<td>8</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDH (matrix)</td>
<td>1260</td>
<td>1464</td>
<td>1546</td>
<td>913</td>
<td>3635</td>
</tr>
<tr>
<td>Sp. activity</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-subunit</td>
<td>100</td>
<td>210</td>
<td>33</td>
<td>200</td>
<td>262</td>
</tr>
<tr>
<td>Rel. spec. ir.</td>
<td>2</td>
<td></td>
<td></td>
<td>19</td>
<td>79</td>
</tr>
</tbody>
</table>

1990
Mitochondrial cyclic AMP-dependent protein kinases

Table 4. Cyclic AMP-dependent protein kinase activities in liver cytosol and mitochondria

<table>
<thead>
<tr>
<th>Additional treatments</th>
<th>Cytosol (pmol/min per mg of protein)</th>
<th>Mitochondria (pmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3868</td>
<td>1249</td>
</tr>
<tr>
<td>Proteinase inhibitors</td>
<td>3519</td>
<td>1099</td>
</tr>
<tr>
<td>NaF (20 mm)</td>
<td>3991</td>
<td>1322</td>
</tr>
<tr>
<td>Freeze-thaw</td>
<td>4095</td>
<td>1230</td>
</tr>
<tr>
<td>Sonication</td>
<td>62</td>
<td>661</td>
</tr>
</tbody>
</table>

Further increased by addition of different proteinase inhibitors, of NaF as a phosphatase inhibitor, or by freezing-thawing of the samples. Sonication destroyed the kinase activity. In sub-fractionated mitochondria, only low cyclic AMP-dependent protein kinase activities were measured in the mitoplast and inner-membrane fractions (Table 4). Some activity measured in the outer-membrane/intermembrane fraction (results not shown) was apparently caused by contamination with cytosol (see Table 3). The matrix fractions, however, showed an increased cyclic AMP-dependent protein kinase activity of twice the value found in the mitochondria (Table 4). This increase in the kinase activity correlated with the 2-fold increase in the specific amount of the catalytic subunit as determined by immunoblotting (Fig. 5, Table 3).

DISCUSSION

Immunolabelling of cyclic AMP-dependent protein kinase subunits in situ and in fractionated tissue indicated the presence of a high concentration of the kinase in mammalian mitochondria. This seemed not to be caused by cross-reactivity of the antisera with other mitochondrial proteins. Immunoblots showed highly specific reactions and the immuno-electron-microscopy results obtained with the three different antisera against the C-, RI- and RII-subunits of cyclic AMP-dependent protein kinases were qualitatively and quantitatively similar.

The predominance of RI-subunit immunoreactivity indicated a preferential occurrence of the type I cyclic AMP-dependent protein kinase in rat heart and pancreas mitochondria. On the other hand, both the type I and the type II kinases seemed to be present in rat liver mitochondria as demonstrated by the relative equivalence of RI- and RII-subunit immunoreactivity. This is in agreement with the results of others [46,47], who determined by non-immunological methods the ratio of the type I/type II enzyme of 4:1 in rat heart and of 3:2 in rat liver extracts. It also indicates that there is neither a species- or tissue-specific [46,47] nor an organelle-specific distribution of the two cyclic AMP-dependent protein kinase isoenzymes in mammalian tissues.

The high concentration of mitochondrial cyclic AMP-dependent protein kinase (4-fold that in the cytoplasm or cytosol) found by the immunological methods could be due to a better preservation and/or immunoreactivity of mitochondrial C- and R-subunits. However, it could also be indicative of the special significance of cyclic AMP-dependent protein kinases in the regulation of mitochondrial processes. A high cyclic AMP-binding activity was also found in mitochondria from yeast; it was also mainly localized at the inner membrane [48].

In accordance with our immunoblot data, the specific activity of cyclic AMP-dependent protein kinase was increased 2-fold in the mitochondrial-matrix fraction. The value obtained (60% of the cytosolic specific activity) was considerably higher than those previously reported for liver mitochondria [12,13]. However, it was still lower than expected from the immunological data. One of the main reasons for this discrepancy seems to be insufficient solubilization of membrane-bound kinases, even in the presence of Triton X-100. This is also indicated by the small amounts of kinase activity measured in the mitoplasts from which the matrix was prepared. The probably more efficient solubilization conditions used in the immunochemical determinations (6 M-urea and sonication for the e.l.i.s.a., SDS/PAGE for immunoblotting) could not be applied, since they destroyed the enzyme activity.

A further explanation for previous difficulties in localizing cyclic AMP-dependent protein kinase activity in mitochondria could be found in the specific spatial arrangement of the enzyme in the inner-membrane/intermembrane space. The results of our immunochemical determinations in sub-mitochondrial fractions suggested that the RI-subunit is bound mainly to the inner membrane. The more variable distribution of C-subunit between both the inner membrane and the matrix fraction in the same preparations suggests a looser association of C-subunit with the inner membrane, possibly through binding to the R-subunit. The
experimental procedure could result in a separation of C-subunit from the membrane-bound R-subunit, and therefore in a loss of cyclic AMP-dependence of the kinase activity. On the other hand, the cyclic AMP-dependent character of mitochondrial protein kinase activities was often decided in previous studies [10–13] on the basis of the stimulatory effect of cyclic AMP.

The present results on the localization of cyclic AMP-dependent protein kinases in mitochondrial subfractions are in accordance with the results of Burgess & Yamada [14], who found in differentially fractionated heart mitochondria about 80 % of the mitochondrial cyclic AMP-dependent protein kinase activity in the matrix compartment. They also fit observations that incubation of isolated liver mitochondria followed by analysis of submitochondrial fractions suggested accumulation of a significant portion of cyclic AMP in the matrix [49], and that phosphodiesterase activity was present in mitochondrial inner membranes [50]. In addition, different mitochondrial enzymes, which have been found in vitro to be substrates of cyclic AMP-dependent protein kinases, are localized either in the matrix or at the inner membrane. These include glutamate dehydrogenase [51], NAD-dependent isocitrate dehydrogenase and succinic dehydrogenase [52], transhydrogenase [53] and side-chain-cleavage cytochrome P-450 [54]. Further studies are necessary to unravel the precise role of cyclic AMP-dependent protein kinases in different mitochondrial functions localized in the inner membrane/matrix space, such as gene expression, oxidative phosphorylation and anion transport.

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