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

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Observation and quantification of the catalytic subunit C of cyclic AMP-dependent protein kinases by immuno-gold electron microscopy suggested a high concentration of cyclic AMP-dependent protein kinases in mitochondria from liver, kidney, heart and skeletal muscle, pancreas, parotid gland and brain cells. The position of gold particles pointed to a localization in the inner membrane/matrix space. A similar distribution was obtained by immunolocalization of the cyclic AMP-dependent protein kinase regulatory subunits RI and RII in liver, pancreas and heart cells. The results indicated the presence of both the type I and the type II cyclic AMP-dependent protein kinases in mitochondria of hepatocytes, and the preferential occurrence of the type I protein kinase in mitochondria from exocrine pancreas and heart muscle. The immunocytochemical results were confirmed by immunochemical determination of cyclic AMP-dependent protein kinase subunits in fractionated tissues. Determinations by e.l.i.s.a. of the C-subunit in parotid gland cell fractions indicated about a 4-fold higher concentration of C-subunit in the mitochondria than in a crude 1200 g supernatant. Immunoblot analysis of subfractions from liver mitochondria supported the localization *in situ* of cyclic AMP-dependent protein kinases in the inner membrane/matrix space and suggested that the type I enzyme is anchored by its regulatory subunit to the inner membrane. In accordance with the immunoblot data, the specific activity of cyclic AMP-dependent protein kinase measured in the matrix fraction was about twice that measured in whole mitochondria. These findings indicate the importance of cyclic AMP-dependent protein kinases in the regulation of mitochondrial functions.

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 Ca^{2+} 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*, benzamide, L-1-tosylphenylalanylchloromethane ('TPCK'), aprotinin, Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, Lubrol and RNA were from Sigma (Deisenhofen, Germany). NADH, oxalacetate, phenylmethanesulphonyl 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 kinase 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/mmol) was from New England Nuclear (Dreieich, Germany), and [^{125}I]-Protein A and [^{14}C]-labelled marker proteins were from Amersham (Braunschweig, Germany). Protein A labelled with 15 nm gold particles was purchased from Janssen (Beerse, Belgium), and alkaline phosphatase-conjugated goat anti-rabbit IgG was from Dianova (Hamburg, Germany). Other chemicals were of reagent grade and were obtained from Merck (Darmstadt, Germany). Microtitre plates were from Nunc (Wiesbaden-Biebrich, Germany), nitrocellulose paper was from Schleicher and Schuell (Dassel, Germany), and Centricon 10 tubes were from Amicon (Danvers, MA, U.S.A.).

The heat-stable inhibitor of cyclic AMP-dependent protein kinases was prepared from bovine muscle as described in ref. [18]. Cyclic AMP-dependent protein kinase subunits were purified [19–21] from bovine (C, RII) or rat (RI) heart, and antisera against them were produced and characterized as described previously [22,23].

Animals

Male Wistar rats (180–210 g) were obtained from Mus-Rattus G.m.b.H. (Brunnthal, Germany) and maintained on standard chow. The rats were anaesthetized with pentobarbital (60 mg/kg body wt.), and for the biochemical and immunochemical determinations were killed by heart incision. Liver and parotid glands were removed, and the latter were also micro-dissected under a stereo-microscope in oxygenated Krebs–Ringer buffer [24].

Preparation of mitochondria and submitochondrial fractions

Mitochondria from micro-dissected rat parotid glands were prepared by a sequence of centrifugation steps on high-density sucrose solutions containing 4% (w/v) Ficoll-400 as described by Cameron & Castle [25].

Mitochondria from rat liver were prepared from a 5% (w/v) homogenate in buffer (10 mM-imidazole, 4 mM-MgCl₂, 0.15 M-NaCl, 1 mM-dithioerythritol, 0.5 mM-phenylmethanesulphonyl fluoride, pH 7.4) containing 0.25 M-sucrose by differential centrifugation as described by Sottocasa *et al.* [26]. Mitoplasts were separated from an outer-membrane/intermembrane fraction by treatment with digitonin (2 mg/10 mg of protein) and centrifugation for 10 min at 12000 *g* (r_{av} 7 cm) as described by Schnaitmann *et al.* [27]. Inner-membrane and matrix fractions were separated from the mitoplasts by treatment with Lubrol (0.16 mg/mg of protein) and centrifugation for 50 min at 144000 *g* (r_{av} 5.9 cm) [28]. Fractions containing the outer-membrane/intermembrane space or the matrix were concentrated 2–3-fold in Centricon tubes. In part of the experiments, 5 mM-benzamidine, 0.1 mM-TPCK and 4 μg each of leupeptin, antipain and pepstatin/ml were included as proteinase inhibitors in addition to 0.5 mM-phenylmethanesulphonyl fluoride.

For morphological characterization, the mitochondrial fraction was fixed with 2% (v/v) glutaraldehyde, post-fixed with 1% (w/v) OsO₄ and embedded in Spurr resin. Thin sections (50–70 nm) were stained in uranyl acetate and lead citrate.

Immuno-electron microscopy

Rats were anaesthetized as described above and tissues were fixed by vascular perfusion [29] with 1.25% (v/v) glutaraldehyde dissolved in oxygenated Krebs–Ringer buffer containing 2% (w/v) polyvinylpyrrolidone for 8–9 min. Tissues were dissected,

trimmed into 1 mm³ pieces and immersed for a further 2 h in 1.25% (v/v) glutaraldehyde/2% (w/v) polyvinylpyrrolidone, dissolved in 0.1 M-potassium phosphate (pH 7.4)/0.15 M-NaCl/0.02% NaN₃. The tissue pieces were dehydrated and embedded in Lowicryl K4M as previously described [30].

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-II), 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 $\times 22\,500$. The areas were determined in mitochondria by the point-counting method [32], and in other cell compartments by randomly superimposed transparent standards of 1 μm^2 .

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 [^{32}P]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.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 micro-titre plate wells, and binding of antibodies from C-antiserum, 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 M-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 [^{125}I]-Protein A (1.15 $\mu\text{Ci}/10\text{ ml}$) and autoradiography. [^{14}C]-labelled *M_r* 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

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 $\times 31\,500$. Bar represents $0.5\ \mu\text{m}$.

as in [41]. Protein was measured as described in [42], with BSA as standard.

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 $1\ \mu\text{m}^2$ each (cytoplasm, nuclei) were evaluated. The values are means $\pm$ S.E.M. The labelling density in the intercellular space was 0–1 gold particles/ μm^2 . 'Cytoplasm' includes cytoplasmic structures with the exception of mitochondria and granules.

Cell type	Cell compartment ...	No. of gold particles/ μm^2			n
		Mitochondria	Cytoplasm	Nucleus	
Hepatocyte		49.5 ± 2.7	19.1 ± 1.1	20.3 ± 1.1	18
Pancreatic acinar cell		36.7 ± 5.2	8.7 ± 1.3	7.8 ± 0.7	8
Parotid acinar cell		47.0 ± 6.0	11.5 ± 2.1	10.2 ± 1.3	39
Cardiomyocyte		39.2 ± 7.3	29.5 ± 5.1	15.1 ± 2.8	5
Skeletal-muscle myocyte		42.8 ± 4.1	23.4 ± 1.6	11.9 ± 0.6	10
Kidney cell (pars convoluta)		37.8 ± 4.2	13.5 ± 2.0	11.7 ± 1.2	10
Nerve cell (cortex)		59.6 ± 7.9	22.3 ± 3.9	22.4 ± 3.8	13

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, $\times 54000$; other panels, $\times 66400$. Bar represents $0.2\ \mu\text{m}$.

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.

Immunoreactivity	Cell type	Cell compartment...	No. of gold particles/ μm^2				n
			Mitochondria	Cytoplasm	Nuclei	Intercellular space	
Anti-RI	Hepatocyte		18.8 ± 1.2	8.5 ± 0.8	3.7 ± 0.3	1.1 ± 0.3	15
	Pancreatic acinar cell		23.0 ± 1.7	11.1 ± 1.0	5.1 ± 0.6	0.3 ± 0.2	20
	Cardiomyocyte		21.6 ± 0.9	12.1 ± 0.7	3.3 ± 0.3	0.4 ± 0.1	20
Anti-RII	Hepatocyte		22.9 ± 2.4	16.8 ± 1.7	12.7 ± 2.1	5.0 ± 0.7	10
	Pancreatic acinar cell		6.6 ± 1.5	4.4 ± 0.9	3.6 ± 0.5	4.4 ± 0.8	15
	Cardiomyocyte		4.3 ± 0.8	2.6 ± 0.4	1.2 ± 0.2	1.2 ± 0.2	5

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/ OsO_4 -fixed samples were examined in the electron microscope. Magnification $\times 6000$. Bar represents $2 \mu\text{m}$.

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 (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).

Fig. 4. E.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/A_0 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.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.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 ^{125}I -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 submitochondrial 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 (M_r 49000) appeared to be degraded to the

known [44,45] degradation product of M_r 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 sub-mitochondrial 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

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

Liver mitochondria were prepared, fractionated and characterized by the activities of the marker enzymes lactate dehydrogenase (LDH), monoamine oxidase (MAO), cytochrome c oxidase (cyt. c ox.), succinic dehydrogenase (SDH) and malate dehydrogenase (MDH) as described in the Materials and methods section. The specific activities are given in nmol/min per mg of protein. The LDH activity in the cytosol was 2258 nmol/min per mg of protein. The relative specific activity (RSA) of LDH is the percentage of cytosolic LDH activity present in a cell fraction over the percentage of cytosolic protein in the same fraction. The relative specific immunoreactivity (Rel. spec. ir.) of the C-subunit was calculated by counting the radioactivity of bound ^{125}I -Protein A in C-subunit immunoblots of the sub-mitochondrial fractions (Fig. 5) and relation of the values to that obtained with the whole mitochondria. The percentage values (%) were calculated by taking the sum of the specific activities (or rel. spec. ir.) in the sub-mitochondrial compartments normalized to the proportion of each fraction in terms of total mitochondrial protein [27] and expression of the individual values as percentages of this sum. The data are means from 3 (SDH, LDH), 4 (other marker enzymes) or 5 (C-subunit) experiments. Abbreviations: m., membrane; n.d., not detectable.

	Cell fraction				
	Mitochondria	Mitoplasts	Outer m./interm.	Inner m.	Matrix
LDH (cytosol)					
Sp. activity	142	9	230	6	n.d.
RSA	0.045	0.004	0.087	0.003	—
MAO (outer m.)					
Sp. activity	5	3	18	2	n.d.
%			98	2	—
Cyt. c ox. (inner m.)					
Sp. activity	287	424	44	233	n.d.
%			8	92	—
SDH (inner m.)					
Sp. activity	127	129	6	120	4
%			2	90	8
MDH (matrix)					
Sp. activity	1260	1464	1546	913	3635
%			6	7	87
C-subunit					
Rel. spec. ir.	100	210	33	200	262
%			2	19	79

Fig. 5. Localization by immunoblotting of cyclic AMP-dependent protein kinase C- and R-subunits in subfractions of liver mitochondria

Mitochondria were prepared and fractionated as described in the Materials and methods section. Samples containing 100 μ g (for C subunit) or 50 μ g (for RI subunit) of protein were separated by SDS/PAGE, electroblotted on nitrocellulose paper and probed with anti-C or anti-RI serum. Binding of antibodies was detected by binding of 125 I-labelled Protein A, followed by autoradiography. Lanes: a, mitochondria; b, outer membrane/intermembrane space, c, mitoplasts; d, inner membranes; e, matrix.

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

Liver fractions were prepared as described in the Materials and methods section and characterized as shown in Table 3. Protein kinase activities were measured in Triton-X-100-pretreated samples in the presence of 1.8 μ M-cyclic AMP and with Kemptide as substrate. The values were corrected for cyclic-AMP-independent protein kinase activities measured in the additional presence of the heat-stable inhibitor of cyclic-AMP-dependent protein kinases. Part of the samples was prepared in the presence of additional proteinase inhibitors (see the Materials and methods section) or was treated by freezing (-20°C)-thawing or by sonication (3×5 s at 0°C) before the assay. The values are means from two experiments. In each experiment, the kinase activities were determined in duplicate by using two samples of different protein concentration.

Additional treatments	Cyclic AMP-dependent protein kinase activity (pmol/min per mg of protein)	
	Cytosol	Mitochondria
None	3868	1249
Proteinase inhibitors	3519	1099
NaF (20 mM)	3991	1322
Freeze-thaw	4059	1230
Sonication	62	661

Cyclic AMP-dependent protein kinase activity (pmol/min per mg of protein)			
	Mitoplasts	Inner membranes	Matrix
None	200	226	2175

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/matrix 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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